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(54) **Receptors of interleukin-12 and antibodies.**

(57) This invention relates to Interleukin-12 receptor cDNAs, proteins and uses thereof.

**Fig. 2**

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10      20      30      40      50      60      70
MEPIVTHQVE LLELELSRO GAACRTSECC QDPFPYPOAD SGASAGPRDL RCYRISSDRY ECSWQTEGPT
80      90      100     110     120     130     140
AGVSHFLRCC LSSGRCCYFA AGSATRLQFS DQAGVSVLYT VTLAVESMAR NQTEKSPEVT LQLYNSVRYE
150     160     170     180     190     200     210
PPLGDIKYSR LAGQLRHEWE TPDNGVGAEV QFRHTYFSSP WELGDCGPGD DDTESCLPL DQVAGFQL
220     230     240     250     260     270     280
RRRLGSGQCS SWSKWSIPVC VPPENFPQPG VRFVSVEQLGQ DGRRLTLKE QPTQLPLFG COGLAPGTEV
290     300     310     320     330     340     350
TYRLQLKLS CPCKAKATRT LKLGKRPYLS GAAYNVAVIS SNQPGGGLRQ TWHIPADTHT EPVALNISVG
360     370     380     390     400     410     420
TKGTTNFWPA RAQSHYICIE WQPVGQGGGL ATCSLTAPQD PDPAGMATYS WSRESGANGQ EKCYIITIFA
430     440     450     460     470     480     490
SAMPEDLTLM STVLSTYHFG GNASAACTPH HVSVDHESLD SVSDVWAPSL LSTCPGVLEK YVVRGDED3
500     510     520     530     540     550     560
KQVSEHPVQP TETQVTLGL RAGVAYTVOV RADTANLRGV WSQPORFSIR VQVSDMLIFF ASLGSFLSIL
570     580     590     600     610     620     630
LVGVLTGLL NRAARKLCEP LPTFCASSAI EPPGKETWQ WNPVDFQKE ASLQALVYZ KSHDKGERTF
640     650     660
PLENTLPEE APELALOTEL SLEDGGRCKA KM

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This invention relates generally to cytokine receptors and more specifically relates to Interleukin-12 receptors.

In order for a molecule such as IL-12 to exert its effect on cells, it is now accepted by those skilled in the art that the molecule must interact with molecules, located on cell membranes, referred to as receptors. Patents which exemplify disclosures of interleukin receptors include Honjo et al., U.S. Patent No. 4,816,565; Urdal et al., U.S. Patent No. 4,578,335; Dower et al., U.S. Patent No. 5,180,812; and Taniguchi et al., U.S. Patent No. 5,198,359.

Also soluble forms of interleukin receptors are known (Fanslow, W.C. et al. (1990) Science 248, 739-41). The effect of Interleukin-1 (IL-1) *in vivo* could be regulated via the administration of a soluble form of its receptor. The results that Fanslow report demonstrate the ability of a soluble cytokine receptor (soluble IL-1R) to modulate biological activity upon exogenous administration *in vivo*, presumably by acting as a neutralizing agent for the endogeneously produced, corresponding ligand (IL-1), and provides evidence of the therapeutic potential of soluble cytokine receptors in a variety of clinical disorders. Systemic administration of a soluble, extracellular portion of the receptor for IL-1 (soluble IL-1R) had profound inhibitory effects on the development of *in vivo* alloreactivity. Survival of heterotopic heart allografts was prolonged from 12 days in controls to 17 days in mice treated with soluble IL-1R. Lymph node hyperplasia in response to localized injection of allogeneic cells was completely blocked by soluble IL-1R treatment.

Interleukin-12 (IL-12), formerly known as cytotoxic lymphocyte maturation factor or natural killer cell stimulatory factor, is a 75-KDa heterodimeric cytokine composed of disulfide-bonded 40-KDa (p40) and 35-KDa (p35) subunits that has pleiotropic activities including stimulation of the proliferation of activated T and NK cells (Gately, M. K., et al. (1991) J. Immunol., 147, 874) (Kobayashi, M. et al. (1989) J. Exp. Med., 170, 827), enhancement of the lytic activity of NK/LAK cells (Kobayashi, M., et al., supra; Stern, A.S., et al. (1990) Proc. Natl. Acad. Sci. USA, 87, 6808), enhancement of cytolytic T-cell responses (M. Gately et al. (1992) Cell. Immunology, 143, 127), induction of interferon gamma by resting and activated T- and NK-cells (M. Kobayashi et al., supra; S. H. Chan et al. (1991) J. Exp. Med., 173, 869), and promotion of T<sub>H</sub>1-type helper cell responses (R. Manetti et al. (1993) J. Exp. Med., 177, 1199; C.-S. Hsieh et al. (1993) Science 260, 547).

The biological activity of IL-12 is mediated by the binding of the IL-12 molecules to cell surface, or plasma membrane, receptors on activated T- and NK cells; however, the contributions of the individual subunits, p35 and p40, to receptor binding and signal transduction remain unknown. Studies with labelled IL-12 have shown that this binding occurs in a specific and saturable manner. IL-12 delivers a signal to target cells through a receptor that was initially characterized on PHA-activated CD4+ and CD8+ T-cells and on IL-2 activated CD56+ NK-cells (R. Chizzonite et al. (1992) J. Immunol., 148, 3117; B. Desai, et al. (1992) J. Immunol., 148, 3125). A survey of over 20 human cell lines belonging to the T-, B-, NK- and myelomonocytic lineages only identified a single CD4+, IL-2 dependent human T-cell line (Kit 225) that constitutively expresses the IL-12 receptor and responds to IL-12 (B. Desai, et al. (1992) J. Immunol., 148, 3125; B. Desai, et al. (1993) J. Immunol., 150, 207A). Freshly prepared PHA-activated PBMC and the Kit 225 cell line thus represent two convenient cell sources to study the biochemistry of the functional IL-12 receptor; there may be others. Equilibrium binding experiments with <sup>125</sup>I-labelled IL-12 showed that i) PHA-activated PBMC express several thousand IL-12 receptors which show 3 classes of affinities: high = 5-20 pM, intermediate = 50-200 pM and low = 2-6 nM; ii) IL-12 receptor expression on PBMC is upregulated by mitogen or IL-2 stimulation; and iii) the IL-12 receptor upregulation correlates with the ability of the cells to proliferate in response to IL-12 (R. Chizzonite, et al. (1992) J. Immunol., 148, 3117; B. Desai, et al. (1992) J. Immunol., 148, 3125). It was not clear at this point whether the biologically functional IL-12 receptor consists of one or more subunits. Affinity crosslinking of labelled IL-12 to activated PBMC demonstrated the size of the cell surface IL-12 binding protein(s) under nonreducing conditions to be in the range of about 150 KDa to about 200 KDa. Additional affinity crosslinking and immunoprecipitation experiments with unlabelled IL-12 bound to <sup>125</sup>I-surface labelled activated PBMC identified an IL-12 binding protein that under reducing conditions had a size of about 110 KDa (R. Chizzonite, et al. (1992) J. Immunol., 148, 3117).

The present invention is directed towards an isolated cDNA encoding a human low affinity IL-12 receptor protein or subunit thereof. When expressed in mammalian cells, the cDNA gives rise to substantially homogeneous IL-12 receptor protein that binds IL-12 in a specific and saturable manner with an apparent affinity of about 2-10 nM, preferably 2-5 nM. Further, this invention relates to novel antibodies against the interleukin-12 receptor. Representative anti-interleukin-12 antisera provided in accordance with the present invention block interleukin-12 binding to cells expressing interleukin-12 receptors, and can also neutralize interleukin-12 activity.

## Brief description of the drawings:

Figure 1: DNA sequence of human IL-12 receptor cDNA clone No. 5. (translated portion = nucleotides 65 to 2050) (SEQ ID NO:1).

Figure 2: Amino acid sequence of human IL-12 receptor protein as deduced from cDNA sequence of Figure 1. (underlined amino acid residues of N-terminal sequence = signal peptide sequence; amino acid residues nos. 541 to 571 = transmembrane area marked by -----; 6 potential N-linked glycosylation sites in the extracellular portion marked by -----; conserved areas 1 and 2 of the cytoplasmic domain are marked by

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[amino acid residues nos. 577 to 584 and 618 to 629] (SEQ ID NO:2).

Figure 3: Alignment of the IL-12 receptor protein subunit sequence with human gp130, human granulocyte colony-stimulating factor-receptor (G-CSF-R) and leukemia inhibitory factor-receptor (LIF-R), and resulting consensus sequence. Consensus residues indicated by lowercase letters refer to identities between IL-12 receptor and gp130 only. The following sequence ranges were used: IL-12 receptor protein (SEQ ID NO:2): residues 42-662; gp130: residues 124-742 (Hibi et al. (1990) Cell, 63, 1149); G-CSF-R: residues 98-731 (Fukunaga, et al. (1990) Proc. Natl. Acad. Sci. USA, 87, 8702); LIF-R: residues 331-950 (Gearing, et al. (1991) EMBO J., 10, 2839).

Figure 4A: Scatchard analysis of IL-12 binding to recombinant human IL-12 receptor expressed in COS cells.

Figure 4B: Scatchard analysis of 2-4E6 antibody binding to recombinant human IL-12 receptor expressed in COS cells.

Figure 5: Analysis of the size of the IL-12 receptor protein under reducing conditions. COS cells were transfected with the IL-12 receptor cDNA and labelled with <sup>35</sup>S cysteine as described. Aliquots of cell lysates were immunoprecipitated with different antibodies as follows: Lane 1: negative control Ig; lane 2: 2-4E6 antibody; lane 3: negative isotype matched control antibody 4D6. Marker sizes in KDa are indicated on the left of the figure.

Figure 6: Analysis of the sizes of IL-12 receptor transcripts by RNA blotting. 4 µg of poly A+ RNA were run per lane. Lanes 1-4 and 5-8 represent identical loadings. Lanes 1-4 were probed with a full length cDNA to the receptor, lanes 5-8 with a probe corresponding only to the cytoplasmic portion of the receptor. Lanes 1, 5: unstimulated peripheral blood mononuclear cells (PBMC). Lanes 2, 3, 6, 7: PHA stimulated PBMC. Lanes 1 and 6 represent a 3 day induction of the sample shown in lanes 1 and 5. Samples in lanes 3 and 7 are from an independent 4 day PHA induction and lanes 4 and 8 represent RNA from K6 cells. The bottom panel shows rehybridization of the blots with a probe for ribosomal protein L32 (loading control).

Figure 7: Inhibition of <sup>125</sup>I-IL-12 binding to IL-12 (IL-12R) receptor by mouse anti-IL-12R antiserum

Figure 8: Characterization of the IL-12 binding proteins on IL-12R positive human cells by affinity-crosslinking

Figure 9: Immunoprecipitation of the solubilized <sup>125</sup>I-IL-12/IL-12R crosslinked complex by anti-IL-12R antibodies

Figure 10: Equilibrium binding of <sup>125</sup>I-2-4E6 to PHA-activated PBMC at room temperature

Figure 11: Equilibrium binding of <sup>125</sup>I-2-4E6 to human K6 cells at room temperature

Figure 12: Inhibition of <sup>125</sup>I-2-4E6 binding to K6 cells by purified 2-4E6 (24E6), human IL-12 (HUIL-12) and control antibody (control IgG)

Figure 13: Equilibrium binding of <sup>125</sup>I-IL-12 to human K6 cells at room temperature

Figure 14: Equilibrium binding of <sup>125</sup>I-IL-12 to detergent solubilized IL-12R from K6 cells

Figure 15: Western blot analysis of detergent solubilized IL-12R with mAb 2-4E6

Figure 16: Detection of IL-12 receptor on human cells by flow cytometry

Figure 17: Size of the IL-12 receptor subunit on the surface of transfected COS and CTLL cells. 8% gels were used and marker sizes in KDa are indicated. Lanes 1-4: Analysis of affinity crosslinked complexes under non-reducing conditions. Arrowhead = labelled, uncrosslinked 2-4E6 antibody. Arrows = labelled, uncrosslinked IL-12. Lanes 5-12: Analysis of <sup>125</sup>I-COS Cell Surface Proteins. Sample reduction, binding of 25 nM unlabelled IL-12 to the cells prior to analysis and use of 1 mM DTSSP crosslinker are indicated below the lanes. Lanes 13-16: Analysis of <sup>125</sup>I-CTLL cell surface proteins.

Figure 18: Specific inhibition of IL-12-induced lymphoblast proliferation by a rat anti-IL-12R antiserum. (A) Titration by flow cytometry of anti-COS cell antibodies in an anti-IL-12R antiserum (---) made against 2-4E6-transfected COS cells, preimmune serum (--o--) from the rat used to prepare the anti-IL-12R antiserum, and a rat antiserum made against COS cells transfected with the human type II IL-1R (--■--). (B-D) Effects of rat sera on proliferation of PHA-activated PBMC induced by IL-12 (B), IL-2 (C), or IL-7 (D). All standard errors were <10% of the means.

Figure 19: Equilibrium binding of  $^{125}$ I-IL-12 to COS cells expressing the IL-12 receptor subunit. A) human IL-12 and B) murine IL-12. The insets show analysis of the binding data according to the method of Scatchard.

The present invention is directed to the low affinity receptor of Interleukin-12 (IL-12). The invention includes the homogenous natural IL-12 receptor including allelic variants and subunits as well as functional derivatives thereof: proteins containing a fragment of the natural IL-12 receptor with binding activity for IL-12. Furthermore the present invention includes recombinant IL-12 receptor proteins as well as fusion proteins, i.e. IL-12 receptor derivatives comprising the amino acid sequence of the natural IL-12 receptor or a partial sequence thereof together with amino acid sequences derived from other proteins. The proteins or polypeptides of the present invention have binding activity for IL-12 as measured by standard assays as described below in the Examples.

A preferred aspect of the present invention relates to a human low affinity IL-12 receptor protein or subunit thereof, more preferred the human receptor protein with the amino acid sequence SEQ ID NO:2 or SEQ ID NO:3.

The invention also includes a soluble form of the IL-12 receptor, preferably a protein exhibiting having IL-12 binding activity, said protein having the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:3 or a portion thereof which encodes a truncated IL-12 receptor protein. Soluble forms of the IL-12 receptors can be prepared by methods known in the art: by isolating soluble IL-12 receptor proteins from culture medium, of IL-12 receptor expressing cells chemical or enzymatic cleavage of IL-12 receptor protein or by recombinant DNA technology.

The availability of the purified receptor, preferably in soluble form, presents therapeutic possibilities as well. Addition of soluble IL-12 receptor interferes with the effect of the interleukin on the cells, since the molecule cannot bind to the cell membrane as freely. Hence, an aspect of the invention is the treatment of pathological conditions caused by excess activity of cells possessing IL-12 receptors by adding an amount of soluble IL-12 receptors sufficient to inhibit binding of IL-12 to the aforementioned cells. This methodology can also be modified, and the soluble receptor can be used as a screening agent for pharmaceuticals. Briefly, a pharmaceutical which works as an IL-12 antagonist can do so by blocking the binding of IL-12 to the IL-12 receptor. Prior to determining whether a material would be effective *in vivo*, one may use the purified IL-12 receptor in connection with a potential pharmaceutical to determine if there is binding. If not, then the pharmaceutical may no longer be a desirable candidate. If there is in fact binding, further testing may be indicated.

The IL-12 receptor proteins of the present invention also include non-naturally occurring IL-12 receptor analogous proteins or mutants. These kind of functional derivatives are proteins in which one or more of the amino acids of the natural IL-12 receptor or its fragments have been replaced or deleted without loss of the IL-12 receptor binding activity. Such analogues may be produced by known methods of peptide chemistry or by recombinant DNA technology.

The term IL-12 receptor protein comprises also derivatives which may be prepared from the functional groups occurring as side chains on the residues or the N- or C-terminal groups, by means known in the art, and are included in the invention as long as they remain pharmaceutically acceptable, i.e. they do not destroy the activity of the protein and do not confer toxic properties on compositions containing it. These derivatives may include, for example, polyethylene glycol side-chains which may mask antigenic sites and extend the residence of the Interleukin-12 receptor protein in body fluids. Other derivatives include aliphatic esters of the carboxyl groups, amides of the carboxyl groups by reaction with ammonia or with primary or secondary amines, N-acyl derivatives of free amino groups of the amino acid residues formed with acyl moieties (e.g. alkanoyl or carbocyclic aryl groups) or O-acyl derivatives of free hydroxyl groups (for example that of seryl- or threonyl residues) formed with acyl moieties.

The present invention also relates to cloned DNA sequences coding for the IL-12 receptor and to polynucleotides encoding a protein as defined above, which polynucleotide contains a sequence corresponding to the cDNA encoding the IL-12 receptor, to recombinant vectors comprising a polynucleotide encoding an IL-12 receptor protein, to microorganisms transformed with said recombinant vectors as well as to processes for the preparation of said proteins, polynucleotides and vectors.

The term "polynucleotide containing a sequence corresponding to the cDNA encoding the IL-12 receptor" refers to a polynucleotide containing a sequence which is homologous to or complementary to a sequence in the sequence encoding the IL-12 receptor. The degree of homology to the cDNA will be at least about 80%, preferably at least about 90%.

5 The practice of the present invention will employ, unless otherwise indicated conventional techniques of molecular biology, microbiology, recombinant DNA and immunology, which are within the skills of an artisan in the field. Such techniques are explained fully in the literature. See e.g. Sambrook, Fritsch & Maniatis, Molecular Cloning; a laboratory Manual (1989) Cold Spring Harbor Laboratory Press 1989.

10 The DNA sequences and DNA molecules of the present invention may be expressed using a wide variety of host vector combinations. For example, useful expression vectors may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Examples of such vectors are viral vectors, such as the various known derivatives of SV40, bacterial vectors, such as plasmids from *E. coli*, phage DNAs, such as the numerous derivatives of phage  $\lambda$ , M13 and other filamentous single-stranded DNA phages, as well as vectors useful in yeasts, such as the 2 $\mu$  plasmid, vectors useful in eukaryotic cells more preferably vectors useful in animal cells, such as those containing SV40, adenovirus and/or retrovirus derived DNA sequences.

20 An aspect of the present invention is a process for preparing a DNA molecule coding for the low affinity Interleukin-12 receptor as described above, characterized in a) the preparation of an anti-Interleukin-12 receptor antibody, b) screening of expression products of a cDNA library, with said antibody, c) identifying and isolating the Interleukin-12 receptor cDNA and d) if desired, preparing a DNA molecule which codes for a functional derivative. A further aspect of the present invention is a process for preparing a low affinity Interleukin-12 receptor protein as described above, characterized in that a) a cell is transformed with a vector comprising a clones gene coding for a low affinity Interleukin-12 receptor protein, b) expression of said protein, c) recovering of said protein and, if desired converting it into a functional derivative thereof.

25 As used herein, "DNA sequence" refers to a DNA polymer, in the form of a separate fragment or as a component of a larger DNA construct, which has been derived from DNA isolated at least once in substantially pure form, i.e., free of contaminating endogenous materials and in a quantity or concentration enabling identification, manipulation, and recovery of the sequence and its component nucleotide sequences by standard biochemical methods, for example, using a cloning vector. Such sequences are preferably provided in the form of an open reading frame uninterrupted by internal nontranslated sequences, or introns, which are typically present in eukaryotic genes. However, it will be evident that genomic DNA containing the relevant sequences could also be used. Sequences of non-translated DNA may be present 5' or 3' from the open reading frame, where the same do not interfere with manipulation or expression of the coding regions.

35 As used herein, "expression vector" refers to a plasmid comprising a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences. Structural elements intended for use in various eukaryotic expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an N-terminal methionine residue. This residue may optionally be subsequently cleaved from the expressed recombinant protein to provide a final product.

45 The host cell used for the expression of DNA sequence may be selected from a variety of known hosts. Examples for such hosts are prokaryotic or eukaryotic cells. A large number of such hosts are available from various depositories such as the American Type Culture Collection (ATCC) or the Deutsche Sammlung für Mikro-organismen (DSM). Examples for prokaryotic cellular hosts are bacterial strains such as *E. coli*, *B. subtilis* and others. Preferred hosts are mammalian cells such as the SV40 transformed African Green monkey kidney cell line COS.

50 The IL-12 receptor protein produced by fermentation of the prokaryotic and eukaryotic hosts transformed with the DNA sequences of this invention can then be purified to essential homogeneity by known methods such as, for example, by centrifugation at different velocities, by precipitation with ammonium sulphate, by dialysis (at normal pressure or at reduced pressure), by preparative isoelectric focusing, by preparative gel electrophoresis or by various chromatographic methods such as gel filtration, high performance liquid chromatography (HPLC), ion exchange chromatography, reverse phase chromatography and affinity chromatography (e.g. on Sepharose™ Blue CL-6B or on carrier-bound monoclonal antibodies).

55 The size of the IL-12 receptor subunit at the cell surface was estimated by affinity crosslinking of labelled IL-12 as well as cell-surface labelling studies. Transfected COS cells express the IL-12 receptor

subunit as a protein of about 100 KDa size. The calculated molecular weight for the mature form of the protein is 70,426; thus, about 25% of the molecular weight of the surface expressed protein is likely to be carbohydrate. Transfected COS cells also express a larger molecular weight form of the IL-12 receptor subunit.

5 The available evidence supports the conclusion that the IL-12 receptor dimerization/oligomerization is independent of IL-12 binding. Similar to these findings, it has been reported for the EPO receptor that disulfide-bonded receptor dimers and oligomers are formed and that EPO stimulation had no detectable effect on receptor dimerization (O. Miura, et al. (1993) Archives Biochem. Biophys., 306, 200). Our data also indicate that only the IL-12 receptor dimers/oligomers bind IL-12 with the 2-5 nM affinity observed on intact  
10 transfected COS cells. i) An anti-IL-12 antibody only immunoprecipitates an affinity crosslinked complex corresponding to one IL-12 and a receptor dimer/oligomer. ii) Affinity crosslinked complexes of the size expected for one receptor subunit and one IL-12 are formed very inefficiently at IL-12 concentrations corresponding to the  $K_D$  measured on transfected COS cells. iii) Murine CTLL cells stably expressing the receptor subunit bind IL-12 very inefficiently (estimated  $K_D$  = 50 nM or lower); these cells also do not  
15 express subunit dimers/oligomers. It was unexpected to find that COS cells and CTLL cells differ in their ability to express the IL-12 receptor subunit in a way that allows IL-12 binding. This could be due to species specificity: murine CTLL cells are somehow unable to "process" the human IL-12 receptor protein correctly, resulting in inefficient dimerization/oligomerization and IL-12 binding. Conceivably, COS cells could express an endogenous protein that allows the IL-12 receptor dimerization/oligomerization to occur. Since under the  
20 experimental conditions used, the number of low-affinity IL-12 receptor sites per transfected COS cell is always greater than  $10^5$ , it seems unlikely that an endogenous COS cell component forms dimers or oligomers with the receptor subunit.

The IL-12 receptor subunit is a member of the hemopoietin receptor superfamily. Within that family, it is most closely related over its entire length to gp130 and the receptors for G-CSF and LIF. The extracellular  
25 portion of the IL-12 receptor subunit can also be divided into five fibronectin type III repeats, similar to what was reported for gp130 (M. Hibi, et al. (1990) Cell, 63, 1149). It is interesting to note that the ligands for IL-12 receptor and gp130, i.e. IL-12 p40 and IL-6 receptor, both also contain such fibronectin type III repeats (Hibi, et al., supra; Schoenhaut, et al. (1992) J. Immunol., 148, 3433). Some features of the cytoplasmic portion of the IL-12 receptor subunit merit further comment. Compared to the corresponding areas in gp130  
30 (276 AA) and the receptor for LIF (237 AA), it is rather short. However, mutagenesis studies have shown that for gp130, only about 100 amino acids in the cytoplasmic region are sufficient to transduce a signal (Murakami, et al. (1991) Proc. Natl. Acad. Sci. USA, 88, 11349). The potential functionality of the IL-12 receptor cytoplasmic portion appears to be borne out further by the presence of a number of features conserved in other functional hemopoietic receptors (among them the receptors for G-CSF, EPO and GM-CSF): conserved areas 1 and 2 (Murakami, et al. (1991) Proc. Natl. Acad. Sci. USA, 88, 11349) are clearly  
35 present and thus give the low affinity IL-12 receptor component the makeup of a beta type subunit (N. Stahl, et al., 1993, Cell, 74:587).

The IL-12 receptor cDNA is useful for the following purposes:

Expression of recombinant IL-12 receptor protein in high levels and its use as an antigen allows  
40 production of additional neutralizing monoclonal and polyclonal antibodies. Such neutralizing antibodies can be used in *in vivo* model settings to elucidate the role that IL-12 and its receptor play in normal as well as pathologic immune responses (i.e. disease states that are aggravated by activated T- and NK-cells like autoimmune diseases, graft versus host disease and rheumatoid arthritis).

IL-12 receptor proteins can be administered, for example, for the purpose of suppressing immune  
45 responses in a human. A variety of diseases or conditions are caused by an immune response to alloantigen, including allograft rejection and graft-versus-host reaction. In alloantigen-induced immune responses, IL-12 receptor can suppress lymphoproliferation and inflammation which result upon activation of T cells. IL-12 receptor can therefore be used to effectively suppress alloantigen-induced immune responses in the clinical treatment of, for example, rejection of allografts (such as skin, kidney, and heart transplants),  
50 and graft-versus-host reactions in patients who have received bone marrow transplants.

IL-12 receptor can also be used in clinical treatment of autoimmune dysfunctions, such a rheumatoid arthritis, diabetes and multiple sclerosis, which are dependent upon the activation of T cells against antigens not recognized as being indigenous to the host. IL-12 receptor can also be useful in treatment of septic shock in which interferon gamma produced in response to IL-12 plays a central role in causing morbidity  
55 and mortality (Doherty et al. (1992) J. Immunol. 149, 1666).

Purified IL-12 receptor compositions will be useful in diagnostic assays for IL-12 or IL-12 receptor, and also in raising antibodies to IL-12 receptor for use in diagnosis or therapy. In addition, purified IL-12 receptor compositions may be used directly in therapy to bind or scavenge IL-12, thereby providing a

means for regulating the immune or inflammatory activities of IL-12. In its use to prevent or reverse pathologic immune responses, soluble IL-12 receptor can be combined with other cytokine receptor antagonists such as antibodies to the IL-2 receptor, soluble TNF receptor, the IL-1 receptor antagonist, and the like.

5 The dose ranges for the administration of the IL-12 receptor proteins and fragments thereof may be determined by those of ordinary skill in the art without undue experimentation. In general, appropriate dosages are those which are large enough to produce the desired effect, for example, blocking the binding of endogenous IL-12 to its natural receptor. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will  
10 vary with the age, condition, sex and extent of disease in the patient, counter indications, if any, immune tolerance and other such variables, to be adjusted by the individual physician. The expected dose range is about 1 ng/kg/day to about 10 mg/kg/day. The IL-12 receptor proteins and fragments thereof can be administered parenterally by injection or by gradual perfusion over time. They can be administered intravenously, intraperitoneally, intramuscularly, or subcutaneously.

15 Preparations for parenteral administration include sterile or aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated  
20 Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, anti-microbials, anti-oxidants, chelating agents, inert gases and the like. See, generally, *Remington's Pharmaceutical Science*, 18th Ed., Mack Eds., 1990.

The present invention also relates to novel immunoglobulins, which include novel antisera and  
25 monoclonal antibodies to the human IL-12 receptor for all three classes of affinities mentioned above.

Representative anti-IL-12R antisera provided in accordance with the present invention block IL-12 binding to cells expressing IL-12R and can also neutralize IL-12 activity. In further embodiments of the present invention, the monoclonal antibodies which are selective to IL-12R are prepared in accordance with generally known techniques, such as the method of Köhler and Milstein. Suitable monoclonal antibodies to  
30 IL-12R can be modified by known methods to provide chimeric, humanized or single chain antibody (SCA) embodiments.

The immunoglobulins according to the present invention comprise antibodies which are capable of inhibiting the binding of IL-12 to the IL-12 receptor and are capable of neutralizing IL-12 bioactivity by binding to the IL-12 receptor. Further the invention refers to immunoglobulins which bind to the IL-12  
35 receptor but which are not capable of inhibiting the binding of IL-12 to the IL-12 receptor and are not capable of neutralizing IL-12 bioactivity by binding to the IL-12 receptor.

The IL-12 receptor antibodies of the present invention can be used to determine IL-12 receptor expression on human cells, such as peripheral blood lymphocytes and bone marrow cells, in normal and pathological conditions. The antisera and monoclonal antibodies of the invention can also be used to block  
40 IL-12 binding to its receptor and thus block its biologic activity. Neutralizing antibodies of the present invention can thus be used for therapeutic intervention in a number of disease states that are aggravated by activated T-cells and NK cells, such as autoimmune diseases, graft versus host disease and rheumatoid arthritis. Finally, as has been specifically demonstrated by the specific antibody embodiment of the present invention, such antibody will also be useful for expression cloning strategies to isolate a cDNA coding for  
45 the IL-12 receptor.

The antisera of the invention can be conveniently produced by immunizing host animals with PHA-activated human PBMC. Suitable host animals include rodents, such as, for example, mice, rats, rabbits, guinea pigs and the like, or higher mammals such as goats, sheep, horses and the like. Initial doses and booster shots can be given according to accepted protocols for eliciting immune responses in animals, e.g.,  
50 in a preferred embodiment mice received an initial dose of  $6 \times 10^7$  cells/mouse i.p. and five subsequent booster shots of between  $2.5 \times 10^7$  cells over a six month period. Immunized mice were observed to develop an immune response against the human IL-12R as determined by inhibition of  $^{125}\text{I}$ -IL-12 binding to PHA-activated PBMCs (Figure 7) and immunoprecipitation of the complex of  $^{125}\text{I}$ -IL-12 crosslinked to IL-12R, which methods provide a convenient way to screen for hosts which are producing antisera having the  
55 desired activity.

Monoclonal antibodies are produced conveniently by immunizing Balb/c mice according to the above schedule followed by injecting the mice with  $1 \times 10^7$  cells i.p. and  $2.5 \times 10^6$  cells iv. on two successive days starting four days prior to the cell fusion. Other protocols well known in the antibody art may of course be



utilized as well. The complete immunization protocol detailed herein provided an optimum protocol for serum antibody response for the antibody to the human IL-12 receptor. Other immunization protocols resulted in a lower serum antibody response than the protocol set forth below: For example, 1) immunization with lower numbers of PHA-activated lymphoblasts ( $0.7$  to  $1.8 \times 10^7$  cells/booster immunization); 2) immunization with fewer numbers of booster immunization or over a short period of time (40 days) with  $2$  to  $6 \times 10^7$  cells/immunization; and 3) immunizations with cell membranes derived from PHA-activated lymphoblasts (membranes equivalent to  $1$  to  $4 \times 10^8$  cells/immunization) all produced serum antibody responses but not as significant as the protocol below. Similar results were obtained when immunizing rats.

B lymphocytes obtained from the spleen, peripheral blood, lymph nodes or other tissue of the host may be used as the monoclonal antibody producing cell. Most preferred are B lymphocytes obtained from the spleen. Hybridomas capable of generating the desired monoclonal antibodies of the invention are obtained by fusing such B lymphocytes with an immortal cell line, that is a cell line that which imparts long term tissue culture stability on the hybrid cell. In the preferred embodiment of the invention the immortal cell may be a lymphoblastoid cell or a plasmacytoma cell such as a myeloma cell, itself an antibody producing cell but also malignant. Murine hybridomas which produce IL-12R monoclonal antibodies are formed by the fusion of mouse myeloma cells and spleen cells from mice immunized against hIL-12R expressed on the surface of activated peripheral blood mononuclear cells. Chimeric and humanized monoclonal antibodies can be produced by cloning the antibody expressing genes from the hybridoma cells and employing recombinant DNA methods now well known in the art to either join the subsequence of the mouse variable region to human constant regions or to combine human framework regions with complementarity determining regions (CDR's) from a donor mouse or rat immunoglobulin, EP 0239400). An improved method for carrying out humanization of murine monoclonal antibodies which provides antibodies of enhanced affinities is set forth in International Patent Application No. WO 92/11018.

The invention also includes a process for the preparation of a monoclonal antibody directed to an Interleukin-12 receptor which comprises a) immunizing a host animal with Interleukin-12 receptor, b) fusing B lymphocytes of said host animal with an immortal cell line, and c) preparation of monoclonal antibodies by culturing the resulting hybridoma cell line.

Polypeptide fragments comprising only a portion of the primary antibody structure may be produced, which fragments possess one or more immunoglobulin activities. These polypeptide fragments may be produced by proteolytic cleavage of intact antibodies by methods well known in the art, or by inserting stop codons at the desired locations in expression vectors containing the antibody genes using site-directed mutagenesis to produce Fab fragments or  $(\text{Fab}')_2$  fragments. Single chain antibodies may be produced by joining VL and VH regions with a DNA linker (see Huston et. al., Proc. Natl. Acad. Sci. U.S.A., 85, 5879-5883 (1988) and Bird et. al., Science, 242, 423-426 (1988)).

It is also within the skill of the art to utilize the monoclonal antibodies of the present invention as therapeutic agents. They may be formulated for parenteral administration in a manner known in the art such as by dissolving the purified monoclonal antibody product either intact or as a fragment in water for injection and sterile filtering. The dosage form may contain known excipients for parenteral administration of proteins such as buffers, stabilizers and carrier protein. The administered dosage will be selected by the attending physician by giving due consideration to the disease severity and nature as well as the age, size and condition of the patient. As immunoglobulins have demonstrated extended half-lives in patients dosing every 10-14 days is usually sufficient. It is also within the skill of the art to modify the monoclonal antibody by forming a hybrid with a toxin molecule such as with a pseudomonas exotoxin or with the A chain of ricin to provide a hybrid molecule capable of destroying the cells expressing the IL-12R in a selective manner.

The invention also pertains to a method for detecting peripheral blood cells which express the IL-12 receptor, which comprises contacting a sample which contains the subject cells with substances capable of forming complexes with the IL-12 receptors so as to form cellular complexes between the substances and the IL-12 receptors, and detecting such cellular complexes. Another embodiment of the invention provides a method of evaluating cell activity in a subject which comprises detecting peripheral blood cells according to the method described above.

In the preferred embodiments, the substances are capable of forming complexes only with the IL-12 receptors present on the surface of peripheral blood cells in which the receptors were expressed. Particularly preferred are substances which comprise IL-12 monoclonal antibody.

One embodiment of the invention provides a method of evaluating immune cellular activity which comprises:

- a. isolating peripheral blood mononuclear cells;
- b. treating the cells with the IL-12 monoclonal antibody; and
- c. determining the amount of monoclonal antibody bound to the cells.



The invention also involves a method for diagnosing an immune system abnormality in a subject which comprises determining the number of T cells, NK cells, or B-cells in a sample derived from the subject. This method involves contacting the sample with substances capable of forming complexes with the IL-12 receptors and determining the percentage of T cells, NK cells or B cells in the sample which have the IL-12 receptor. Comparing the percentages so determined with the percentage of cells which have the IL-12 receptor in a sample from a normal subject who does not have the immune system abnormality, the differences in the percentage of cells so determined being indicative of the immune system abnormality. Preferably, the subject is an animal, e.g., a human.

As a molecule associated with T cell, NK cell and B cell function, the measurement of IL-12R expression has diagnostic importance. Because IL-12R is distinctive to activated T cells, NK cells or B cells, it is a unique marker for these cells in a population of lymphocytes.

Moreover, the level of expression of IL-12R provides a measure of T cell, NK cell or B cell activity. This information may be important for evaluating the immune status of an individual. For instance, in the treating of certain disease, such as cancer, agents which affect the immunocompetency are often used. Assays for IL-12R expression may allow physicians to monitor the immune status of the patient and to adjust treatment to minimize the risk of opportunistic infection, often a threat to immunocompromised patients.

Assays for IL-12R expression may be conventional immunochemical assays for cell surface antigens. Peripheral blood mononuclear cells can be isolated from patient and incubated with IL-12R monoclonal antibody under conditions which allow the antibody to bind the surface antigen. Antibody bound to the cell surface provides a measure of IL-12R expression. Binding of the antibody to cells may be evaluated by employing an IL-12R monoclonal antibody labelled with a radioactive, fluorescent or other compound capable of being detected.

The invention also involves a method for detecting soluble IL-12 receptor concentration in samples derived from subjects with immune system disorders, cancer, or other diseases that would be marked by an increase or decrease in soluble form of IL-12R. Assays for soluble IL-12R may be conventional sandwich immunochemical assays or  $^{125}\text{I}$ -IL-12 binding assays to immobilized IL-12R.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention. Certain embodiments of this invention are exemplified in the Examples which follow. The Examples are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow.

## MATERIALS AND METHODS

### Proteins and Plasmids

Recombinant human IL-12 (U. Gubler et al., Proc. Natl. Acad. Sci. USA (1991) 88, 4143, and murine IL-12 (D. Schoenhaut et al., J. Immunology (1992), 148, 3433) were obtained as described therein.

The murine anti human IL-12 receptor monoclonal antibody 2-4E6 used herein was generated as described herein below and was purified from ascites fluids by affinity chromatography on protein G-agarose according to the manufacturer's instructions (Genex). The proteins were labelled with I-125 by a modification of the Iodogen method as described (Pierce Chemical Co., Rockford, IL). Radiospecific activities of 5000 - 7000 cpm/fmole for IL-12 and 1500 - 2500 cpm/fmole for the 2-4E6 antibody were typically obtained.

The plasmid pEF-BOS is based on a pUC 119 backbone and contains the elongation factor 1 alpha promoter to drive expression of genes inserted at the BstXI site (S. Mizushima and S. Nagata (1990) Nucl. Acids Res. 18, 5322).

### Example 1

#### Preparation, Characterization and Purification of Hybridoma Antibodies

Balb/c mice (Charles River Laboratories) were immunized by the intraperitoneal route with PHA-(phytohemagglutinin)-activated human PBMC (PHA-activated PBMC) at  $6 \times 10^7$  cells/mouse. Mice received 5 subsequent booster injections of between  $2.5 \times 10^7$  cells over a six month period. For preparation of activated spleen cells, 2 mice were injected intraperitoneally and intravenously with  $1 \times 10^7$  and  $2.5 \times 10^6$

cells, respectively, on two successive days, starting four days prior to the cell fusion. Spleen cells were isolated from these mice and fused with SP2/0 (ATCC CRL 1581) cells at a ratio of 1:1 with 35% v/v polyethylene glycol 4000 (E. Merck) according to the method of Fazekas et al., J. Immunol. Methods (1980) 35, 1. The fused cells were plated at a density of  $6 \times 10^5$  cells/ml/well in 48-well cluster dishes in IMDM supplemented with 10% FBS, glutamine (2 mM),  $\beta$ -mercaptoethanol (0.1 mM), gentamicin (50 g/ml), 5% ORIGEN hybridoma cloning factor (IGEN, Inc.), 5% P388D1 (ATCC CRL 46) supernatant (Nordan, R.P., et al., J. Immunol. (1987) 139, 813) and 100 Units/ml rHuIL-6. Hybridoma supernatants were assayed for specific anti-IL-12 receptor antibodies by: 1) immunoprecipitation of the soluble complex of  $^{125}$ I-HuIL-12 crosslinked to IL-12 receptor (125I-IL-12/IL-12R), 2) inhibition of  $^{125}$ I-HuIL-12 binding to PHA-activated PBMC's, and 3) differential binding to IL-12 receptor positive cells versus receptor negative cells). Hybridoma cell lines secreting specific anti-receptor antibodies were cloned by limiting dilution. Antibodies were purified from ascites fluids by affinity chromatography on Protein G bound to cross-linked agarose according to the manufacturer's protocol (Genex).

## 15 Example 2

### Preparation of Human PHA Lymphoblasts and IL-12 Receptor Binding Assays

Human peripheral blood mononuclear cells were isolated (see Gately et al, J. Natl. Cancer Inst. 69, (1982) 1245) and cultured at 37°C at a density of  $5 \times 10^5$  cells/ml in tissue culture medium (TCM) containing 0.1% PHA-P (Difco). After 3 days, the cultures were split 1:1 with fresh TCM, and human rIL-2 was added to each culture to give a final concentration of 50 units/ml. The cultures were then incubated for an additional 1-2 days, prior to use in assays.

PHA-activated human PBMC were washed once in binding buffer (RPMI-1640, 5% FBS, 25 mM HEPES pH 7.4) and resuspended in binding buffer to a cell density of  $7 \times 10^6$  cells/ml. Lymphoblasts ( $7 \times 10^5$  cells) were incubated with various concentrations of  $^{125}$ I-IL-12 (5-10000 pM) at room temperature for the designated times. Cell bound radioactivity was separated from free  $^{125}$ I-IL-12 by centrifugation of the assay mixture through 0.1 ml of an oil mixture (1:2 mixture of Thomas Silicone Fluid 6428-R15 : A.H. Thomas, and Silicone Oil AR 200 : Gallard-Schlessinger) at 4°C for 90 sec at 10,000 x g. The tip containing the cell pellet was excised, and cell bound radioactivity was determined in a gamma counter. Non-specific binding was determined by inclusion of 100 nM unlabelled IL-12 in the assay. Incubations were carried out in duplicate or triplicate. Receptor binding data were analyzed by using the non-linear regression programs EBDA and LIGAND as adapted for the IBM personal computer by McPherson, J. Pharmacol. Methods (1985) 14, 213 from Elsevier-BIOSOFT.

## 35 Example 3

### Affinity Cross-Linking of $^{125}$ I-IL-12 to IL-12 Receptor Bearing Cell Lines

IL-12 receptor bearing cells were incubated with  $^{125}$ I-IL-12 (100-500 pM) in the presence or absence of excess unlabelled IL-12 for 2 hr at room temperature. The cells were then washed with ice-cold PBS pH 8.3 (25 mM Sodium Phosphate pH 8.3, 0.15 M NaCl and 1 mM  $\text{MgCl}_2$ ) and resuspended at a concentration of  $0.5-1.0 \times 10^7$  cells/ml in PBS pH 8.3. BS3 (Pierce) in dimethyl sulfoxide was added to a final concentration of 0.4 mM. Incubation was continued for 30 min. at 4°C with constant agitation. The cells were washed with ice-cold 25 mM Tris-HCl (pH 7.5), 0.15 M NaCl and 5 mM EDTA and then solubilized at  $0.5 - 1.0 \times 10^8$  cells/ml in solubilization buffer (50 mM Tris-HCl (pH 8.0) containing 8 mM CHAPS, 0.25 M NaCl, 5 mM EDTA, 40  $\mu\text{g/ml}$  PMSF, 0.05%  $\text{NaN}_3$ , and 1% BSA) for 1 hr at 4°C. The extracts were centrifuged at 12,000 x g for 45 min. at 4°C to remove nuclei and other debris.

## 50 Example 4

### Immunoprecipitation Assay of the Soluble Complex of $^{125}$ I-IL-12 Crosslinked to Human IL-12R

For the immunoprecipitation assay, hybridoma culture supernatant (0.5 ml), diluted antisera, or purified IgG was added to a microfuge tube containing 0.1 ml of a 50% suspension of either goat-anti-mouse IgG coupled to agarose (SIGMA CHEM. CO.) or Protein G coupled to Sepharose 4B (Pharmacia). The assay volume was brought up to 1.0 ml with IP buffer (8 mM CHAPS in PBS (0.25 M NaCl), 1% BSA, & 5 mM EDTA) and the mixture was incubated on a rotating mixer for 2 hr at room temperature. The beads were

pelleted by centrifugation, resuspended in 1 ml IP buffer containing  $^{125}\text{I}$ -IL-12/IL-12R (10-20,000 cpm) and the mixture was incubated on a rotating mixer for 16 hr at 4°C. After this incubation, the beads were pelleted by centrifugation and washed twice in IP buffer without BSA. The  $^{125}\text{I}$ -labelled receptor complex bound to the solid phase antibodies was released by adding 100  $\mu\text{l}$  of 2x Laemmli sample buffer (Nature 227, 680 (1970)) with and without 10%  $\beta$ -mercaptoethanol and heating for 5 min. at 95°C. The immunoprecipitated proteins were analyzed by SDS-PAGE on 8% or 4-15% gradient polyacrylamide gels and visualized by autoradiography.

#### Example 5

##### Assays for IL-12R Solubilized from Cells Expressing IL-12 Receptor.

To confirm that the antibodies identified by the immunoprecipitation assay were specific for IL-12R, an immunoprecipitation/soluble IL-12R binding assay was developed. As described in Example 1 above, antibodies (as hybridoma supernatant, purified IgG (50  $\mu\text{g}$ ) or antisera) were immobilized by binding to goat anti-mouse IgG coupled to agarose (100  $\mu\text{l}$ ; Sigma Chemical Co.) or protein G coupled to Sepharose 4B (100  $\mu\text{l}$ ; Pharmacia). For some experiments, antibodies were covalently crosslinked to protein G-Sepharose 4B, before being used in the assay. See Stern and Podlaski, Techniques in Protein Chemistry (1993). The immobilized antibodies were resuspended in IP buffer (0.3 ml) and 0.2 ml of a detergent solubilized extract of PHA-activated PBMCs or K6 cells that contained IL-12R was added. To prepare the detergent solubilized IL-12R preparation, the cells were washed with ice-cold 25 mM Tris-HCl (pH 7.5), 0.15 M NaCl and 5 mM EDTA and then solubilized at  $1.5 \times 10^8$  cells/ml in solubilization buffer (50 mM Tris-HCl, pH 8.0, containing 8 mM CHAPS, 0.25 M NaCl, 5 mM EDTA, 40  $\mu\text{g}/\text{ml}$  PMSF, 0.05%  $\text{NaN}_3$ , and 1% BSA) for 1 hr at 4°C. The extracts were centrifuged at 120,000 x g for 60 min. at 4°C to remove nuclei and other debris. The mixture was incubated on a rotating mixer for 16 hr at 4°C. After this incubation, the beads were pelleted by centrifugation and resuspended in IP buffer (0.15 ml) containing  $^{125}\text{I}$ -HuIL-12 at concentrations ranging from 0.05 to 7.5 nM. The IL-12R immobilized on the antibody coated beads was incubated with  $^{125}\text{I}$ -HuIL-12 for 2 hrs. at room temperature on a shaker. Following this incubation, the beads were pelleted, washed twice with IP buffer and the bound radioactivity determined in a gamma counter. Nonspecific binding was determined by inclusion of 70 nM unlabelled human IL-12 in the assay. Solubilized IL-12R binding data were analyzed according to the method of Scatchard, (Assn. N.Y. Acad. Sci. 51, 660 (1949)) by using the nonlinear regression programs EBDA and Ligand as adapted for the IBM PC by McPherson, supra from Elsevier-BIOSOFT.

#### Example 6

##### Competitive Inhibition of $^{125}\text{I}$ -IL-12 Receptor Binding by Antibodies

The ability of hybridoma supernatant solutions, purified IgG, or antisera to inhibit the binding of  $^{125}\text{I}$ -IL-12 to PHA-activated lymphoblasts was measured as follows: serial dilutions of culture supernatants, purified IgG or antisera were mixed with activated lymphoblasts ( $1-1.5 \times 10^6$  cells) in binding buffer (RPMI-1640, 5% FBS + 25 mM HEPES pH 7.4) and incubated on an orbital shaker for 1 hour at room temperature.  $^{125}\text{I}$ -HuIL-12 ( $1 \times 10^5$  cpm) was added to each tube and incubated for 1-2 hours at room temperature. Nonspecific binding was determined by inclusion of 10 nM unlabelled IL-12 in the assay. Incubations were carried out in duplicate or triplicate. Cell bound radioactivity was separated from free  $^{125}\text{I}$ -IL-12 by centrifugation of the assay through 0.1 ml of an oil mixture as described above. The tip containing the cell pellet was excised, and cell bound radioactivity was determined in a gamma counter.

#### Example 7

##### Labelling of Human IL-12 and Mab 2-4E6 with $^{125}\text{I}$

Human IL-12 and purified 2-4E6 (Example 13) IgG were labelled with  $^{125}\text{I}$  by a modification of the Iodogen method (Pierce Chemical Co., Rockford, IL). Iodogen was dissolved in chloroform and 0.05 mg dried in a 12 x 15 mm borosilicate glass tube. For radiolabeling, 1.0 mCi  $\text{Na}^{125}\text{I}$  (Amersham, Chicago, IL) was added to an Iodogen-coated tube containing 0.05 ml of Tris-iodination buffer (25 mM Tris-HCl pH 7.5, 0.4 M NaCl and 1 mM EDTA) and incubated for 4 min at room temperature. The activated  $^{125}\text{I}$  solution was transferred to a tube containing 0.05 to 0.1 ml IL-12 (7  $\mu\text{g}$ ) or IgG (100  $\mu\text{g}$ ) in Tris-iodination buffer and the

reaction was incubated for 9 min. at room temperature. At the end of the incubation, 0.05 ml of Iodogen stop buffer (10 mg/ml tyrosine 10% glycerol in Dulbecco's PBS, pH 7.40) was added and reacted for 3 min. The mixture was then diluted with 1.0 ml Tris-iodination buffer, and applied to a Bio-Gel P10DG desalting column (BioRad Laboratories) for chromatography. The column was eluted with Tris-iodination buffer, and fractions (1 ml) containing the peak amounts of labelled protein were combined and diluted to  $1 \times 10^8$  cpm/ml with 1% BSA in Tris-iodination buffer. The TCA precipitable radioactivity (10% TCA final concentration) was typically in excess of 95% of the total radioactivity. The radiospecific activity was typically ~ 1500 to 2500 cpm/fmol for 2-4E6 IgG and 5000 to 7000 cpm/fmol for IL-12.

#### 10 Example 8

##### Binding Assays of $^{125}\text{I}$ -2-4E6 to Intact Cells

PHA-activated human PBMC were washed once in binding buffer (RPMI 1640, 5% FBS and 25 mM HEPES, pH 7.4) and resuspended in binding buffer to a cell density of  $1.5 \times 10^7$  cells/ml. Lymphoblasts ( $1.5 \times 10^6$  cells) were incubated with various concentrations of  $^{125}\text{I}$ -2-4E6-IgG (0.005 to 2 nM) at room temperature for 1.5 hrs. Cell bound radioactivity was separated from free  $^{125}\text{I}$ -2-4E6 IgG by centrifugation of the assay mixture through 0.1 ml silicone oil at  $4^\circ\text{C}$  for 90 seconds at  $10,000 \times g$ . The tip containing the cell pellet was excised, and cell bound radioactivity was determined in a gamma counter. Non-specific binding was determined by inclusion of 67 nM unlabelled 2-4E6 IgG in the assay. Incubations were carried out in duplicate or triplicate. Receptor binding data were analyzed by using the nonlinear regression programs EBDA, Ligand and Kinetics as adapted for the IBM personal computer by McPherson, supra from Elsevier BIOSOFT.

#### 25 Example 9

##### Expression of Recombinant IL-12R in COS Cells and Determination of $^{125}\text{I}$ -2-4E6 Binding

COS cells ( $4-5 \times 10^7$ ) were transfected by electroporation with 25  $\mu\text{g}$  of plasmid DNA expressing recombinant human IL-12R, as describe herein below, in a BioRad Gene Pulser (250  $\mu\text{F}$ , 250 volts) according to the manufacturer's protocol. The cells were plated in a 600  $\text{cm}^2$  culture plate, harvested after 72 hours by scraping, washed and resuspended in binding buffer. Transfected cells ( $8 \times 10^4$ ) were incubated with increasing concentrations of  $^{125}\text{I}$ -labelled 2-4E6 or IL-12 at room temperature for 2 hrs. Cell bound radioactivity was separated from free  $^{125}\text{I}$ -labelled 2-4E6 or IL-12 as described above.

#### 35 Example 10

##### Western Blot Analysis of Soluble IL-12R with mAb 2-4E6

PHA-activated PBMC were washed 3 times with ice-cold PBS and solubilized at  $0.5 - 1 \times 10^8$  cells/ml in solubilization buffer (50 mM Tris-HCl pH 8.0 containing 8 mM CHAPS, 0.25 M NaCl, 5 mM EDTA, 40  $\mu\text{g}/\text{ml}$  PMSF, 0.05%  $\text{NaN}_3$  and 1 mg/ml BSA) for 1 hr at  $4^\circ\text{C}$ . The extracts were centrifuged at  $12,000 \times g$  for 45 min. at  $4^\circ\text{C}$  to remove nuclei and other debris. The extracts were incubated with 2-4E6 IgG or control IgG bound to goat-anti-mouse IgG immobilized on cross-linked agarose (Sigma Chemical Co.). The precipitated proteins were released by treatment with 0.1 M glycine pH 2.3, neutralized with 3M Tris, mixed with 1/5 volume of 5 x Laemmli sample buffer, and separated by SDS/PAGE on 8% pre-cast acrylamide gels (NOVEX). The separated proteins were transferred to nitrocellulose membrane (0.2  $\mu\text{M}$ ) for 16 hours at 100 volts in 10 mM TRIS-HCl (pH 8.3), 76.8 mM glycine, 20% methanol and 0.01% SDS. The nitrocellulose membrane was blocked with BLOTTO (5.0% w/v nonfat dry milk in PBS + 0.05% Tween 20) and duplicate blots were probed with  $^{125}\text{I}$ -2-4E6 IgG ( $1 \times 10^6$  cpm/ml in 8 mM CHAPS in PBS, 0.25 M NaCl, 10% BSA and 5 mM EDTA) + unlabelled 2-4E6 IgG (67 nM).

Example 11Analysis of IL-12 Receptor Expression on Human Cells by Fluorescence Activated Cell Sorting with mAb 2-4E6

To stain cells expressing IL-12 receptor;  $1 \times 10^6$  cells in 100  $\mu$ l staining buffer (PBS containing 2% FBS and 0.1%  $\text{NaN}_3$ ) were incubated with 10  $\mu$ l of 2-4E6 ascites fluid for 25 min. at 4°C. Cells were then washed twice with staining buffer followed by incubation with a 1:100 dilution of goat F(ab)2 anti mouse Ig-PE (Tago, Burlingame CA) for 25 min. at 4°C. The stained cells were washed twice with staining buffer and then analyzed on a FACScan flow cytometer (Becton Dickinson).

Example 12Inhibition of IL-12 Binding to Human PHA-Lymphoblasts by Mouse Anti-IL-12R Antiserum

Mice immunized with PHA-activated PBMCs developed an immune response against the human IL-12R as determined by inhibition of  $^{125}\text{I}$ -IL-12 binding to PHA-activated PBMCs (Figure 7) and immunoprecipitation of the complex of  $^{125}\text{I}$ -IL-12 crosslinked to IL-12R (data not shown). The dilutions for half-maximal inhibition of  $^{125}\text{I}$ -IL-12 binding to PHA-activated PBMCs were 1/500 and 1/250 for animals 211-1 and 211-2, respectively (Figure 7). These antisera also neutralized IL-12 biologic activity as measured in a PHA-lymphoblast proliferation assay (data not shown). Spleen cells isolated from these mice were fused with SP2/0 myeloma cells and the resulting hybridomas were initially screened for IL-12R specific antibodies by immunoprecipitation of the  $^{125}\text{I}$ -IL-12/IL-12R complex and by inhibition of  $^{125}\text{I}$ -IL-12 binding to IL-12R.

For Figure 7, ten fold serial dilutions of mouse anti-IL-12R immune serum (#211-1 and #211-2) and normal mouse serum (NMS) were preincubated with PHA-activated PBMC for 60 min at RT (room temperature) before addition of  $^{125}\text{I}$ -IL-12 (100 pM). After addition of  $^{125}\text{I}$ -IL-12, the reaction was incubated for 1-2 hrs at RT and the cell bound radioactivity was determined. The data are expressed as the % inhibition of  $^{125}\text{I}$ -IL-12 binding in the presence of the immune serum when compared to the specific binding in the absence of serum.

Example 13Identification and Characterization of Monoclonal Anti-IL-12R Antibodies

The immunoprecipitation assay identified 13 hybridomas secreting putative non-neutralizing anti-IL-12R antibodies, whereas the IL-12R binding assay identified 3 putative neutralizing IL-12R antibodies (Table 1). The immunoprecipitation assay measured the ability of the putative anti-IL-12R antibodies that are immobilized on a solid phase to capture the solubilized complex of  $^{125}\text{I}$ -IL-12/IL-12R. To verify that the radioactivity immunoprecipitated by the immobilized antibody was present in the complex of  $^{125}\text{I}$ -IL-12/IL-12R, the immunoprecipitated proteins were solubilized, separated by SDS-PAGE and visualized by autoradiography. The preparations of the  $^{125}\text{I}$ -IL-12/IL-12R complexes solubilized from PHA-activated PBMC, Kit-225 and K6 cells were resolved into two major radioactive bands, 210-250 kDa and 75 kDa (Figure 8). The 210-250 kDa and 75 kDa complexes were identified as the  $^{125}\text{I}$ -IL-12/IL-12R complex and  $^{125}\text{I}$ -IL-12 not complexed with the receptor, respectively (Figure 8). See also Chizzonite et al., J. Immunol. 148, 3117 (1992). The radioactive 75 kDa band visualized from the cell extracts co-migrated with  $^{125}\text{I}$ -IL-12, indicating that it represented  $^{125}\text{I}$ -IL-12 that bound but was not covalently crosslinked to IL-12R. The 210-250 kDa band was not a covalent crosslinked oligomer of  $^{125}\text{I}$ -IL-12 because it is not produced when the crosslinking agent BS3 was added directly to  $^{125}\text{I}$ -IL-12 (Figure 8).

Hybridoma cells secreting putative anti-IL-12R antibodies were then cloned by limiting dilution and screened by both the immunoprecipitation and inhibition of binding assays that identify non-neutralizing and neutralizing antibodies, respectively. During this cloning and screening process, hybridoma lines secreting putative neutralizing anti-IL-12R antibodies were not recovered, whereas non-neutralizing antibodies were recovered from both the original immunoprecipitation and inhibitory positive hybridomas. After this initial identification and cloning, a direct binding assay was used to determine if the non-neutralizing antibodies only bound to cells expressing IL-12R. This assay demonstrated that the non-neutralizing antibodies could be divided into 2 classes, those that bound only IL-12R positive human cells and those that bound to most human cells (data not shown). Representative antibodies from each class, 2-4E6 and 2C6, respectively, were produced in ascites fluid, purified by protein G affinity chromatography and extensively characterized.

For Figure 8, PHA-activated PBMC (PHA-PBMC), Kit-225 (Kit-225) and K6 (K6) cells ( $1 \times 10^7$  cells/ml) were incubated with  $^{125}\text{I}$ -IL-12 (100-500 pM) for 2 hrs at room temperature in the absence or presence of 25 nM unlabelled IL-12. Cells were then washed, affinity crosslinked with BS3 (0.4 mM final concentration) and a cell extract prepared as described. The cell extract was precipitated with wheat germ lectin bound to solid supports as described. The precipitated proteins were released by treatment with sample buffer and analyzed by SDS-PAGE and autoradiography on a 8.0% slab gel. The complex of  $^{125}\text{I}$ -IL-12 crosslinked to the IL-12 receptor migrates as a single major band of approximately 210-250 kDa. The band migrating at 75 kDa is  $^{125}\text{I}$ -IL-12 that was bound but not crosslinked to the IL-12 receptor.  $^{125}\text{I}$ -IL-12 (IL-12) and  $^{125}\text{I}$ -IL-12 that was treated with the BS3 crosslinker (IL-12/BS3) were electrophoresed in parallel lanes as markers for the migration of the 75 kDa IL-12 heterodimer and for any oligomers of IL-12 that may form with the BS3 crosslinker. The molecular sizes indicated in the margins were estimated from standards run in parallel lanes. Exposure time was 7 days.

Table 1

INITIAL IDENTIFICATION OF HYBRIDOMAS SECRETING ANTI-IL-12 RECEPTOR ANTIBODIES: SPLENOCYTES FROM MICE #211-1 AND #211-2			
HYBRIDOMA/ANTIBODY I.P. ASSAY <sup>1</sup> (cpm bound)			INHIBITION ASSAY <sup>2</sup>
IL-12R 2C6 <sup>3</sup>			-
211-1	1A5	722	-
	4E6	840	-
	5C1	312	+
211-2	3B1	1323	-
	4A3	2172	-
	4D6	804	-
	5D5	877	-
	4A5	509	+
	4C6	456	+
	1D1	1395	-
	5E6	2043	-
Control mAb 402			-
			-

<sup>1</sup> I.P. assay measures the amount of  $^{125}\text{I}$ -IL-12/IL-12R complex bound by the immobilized antibody.

<sup>2</sup> Inhibition assay measures whether the antibody can inhibit  $^{125}\text{I}$ -IL-12 binding to PHA-activated PBMC.

<sup>3</sup> IL-12R 2C6 is an antibody that both immunoprecipitates the  $^{125}\text{I}$ -IL-12/IL-12R complex and binds to many IL-12R positive and negative human cells. This antibody probably recognizes a component closely associated with the IL-12R.

#### Example 14

##### Characteristics of Monoclonal Anti-IL-12R Antibody 2-4E6 Binding to Natural IL-12R

mAb 2-4E6 immunoprecipitates the  $^{125}\text{I}$ -IL-12/IL-12R complex solubilized from PHA-activated human lymphoblasts, Kit-225 and K6 cells (Figure 9, data shown for PHA-activated PBMC), but does not block  $^{125}\text{I}$ -IL-12 binding to IL-12R expressed on these cells. These data suggested that the 2-4E6 antibody was a non-inhibitory or non-neutralizing anti-IL-12R antibody. To confirm that 2-4E6 was a non-inhibitory antibody specific for the IL-12R, 2-4E6 was labelled with  $^{125}\text{I}$  and direct binding assays were performed with IL-12R positive and negative cells.  $^{125}\text{I}$ -2-4E6 binds to IL-12R bearing cells with an affinity that ranges from 337 pM to 904 pM and identifies between 1500 and 5000 binding sites per cell (PHA-activated PBMC, Figure 10; K6 cells, Figure 11). IL-12 does not block  $^{125}\text{I}$ -2-4E6 from binding to PHA-activated PBMCs and confirms that 2-4E6 is a non-inhibitory/non-neutralizing antibody (Figure 12).  $^{125}\text{I}$ -2-4E6 binds to other cells expressing IL-12R, such as Kit 225, and YT cells, but does not bind to IL-12R negative cells (non-activated human PBMC,

MRC-5 fibroblasts and HL-60 cells (Table 2).

Equilibrium binding assays have demonstrated that  $^{125}\text{I}$ -IL-12 identifies 3 separate binding sites on the surface of PHA-activated PBMCs, Kit-225 and K6 cells (Figure 13, data for K6 cells and Table 2). Analysis of this binding data by the method of Scatchard, supra shows these affinities are approximately 5-20 pM, 50-200 pM and 2-6 nM, respectively. The total number of  $^{125}\text{I}$ -IL-12 binding sites per cell are approximately 1500 to 5000, which is in good agreement with the total number of binding sites identified by  $^{125}\text{I}$ -2-4E6 (Table 2). The data also suggests that 2-4E6 recognizes the low affinity (2-5 nM) binding component of the IL-12 receptor in much the same manner that the anti-TAC antibody recognizes the low affinity component (p55 subunit) of the IL-2 receptor.

Since the data indicated that mAb 2-4E6 was a non-neutralizing antibody specific for the IL-12R, the molecular weight and  $^{125}\text{I}$ -IL-12 binding characteristics of the protein(s) immunoprecipitated by mAb 2-4E6 from the surface of IL-12R positive cells was investigated. The steady state binding of  $^{125}\text{I}$ -IL-12 to proteins immunoprecipitated by immobilized 2-4E6 from solubilized extracts of PHA-activated PBMCs, Kit-225 and K6 cells was saturable and specific (Figure 14, data for extracts from K6 cells). Transformation of the binding data by the method of Scatchard, revealed a single site with an apparent affinity of 188 pM. The proteins immunoprecipitated by 2-4E6 from the cell extracts were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with  $^{125}\text{I}$ -2-4E6 in a western blot. On the western blot,  $^{125}\text{I}$ -2-4E6 binds to an approximately 90 kDa protein, that is only immunoprecipitated by 2-4E6 and not by an anti-IL-12 antibody or a control antibody (Figure 15, data shown for PHA-activated PBMCs). In summary, all the data demonstrated that mAb 2-4E6 bound a protein on the surface of IL-12R positive cells that was approximately 90 kDa and bound  $^{125}\text{I}$ -IL-12 in a specific manner.

For Figure 9, soluble complexes of  $^{125}\text{I}$ -IL-12/IL-12R were prepared from PHA-activated human PBMC as detailed herein (see also Figure 8) and immunoprecipitated by immobilized antibodies, 2-4E6, 2C6, 4D6, 20C2 and control. The soluble complexes were also precipitated with wheat germ lectin immobilized on crosslinked agarose. The precipitated proteins were analyzed as described herein and in Figure 8. Antibodies 4D6 and 20C2 are non-neutralizing and neutralizing anti-IL-12 antibodies, respectively. 4D6 immunoprecipitates  $^{125}\text{I}$ -IL-12:IL-12R complex and free  $^{125}\text{I}$ -IL-12, whereas 20C2 only immunoprecipitates free  $^{125}\text{I}$ -IL-12. Both 2-4E6 and 2C6 recognize the  $^{125}\text{I}$ -IL-12/IL-12R complex.  $^{125}\text{I}$ -IL-12 (IL-12) and  $^{125}\text{I}$ -IL-12 that was treated with the BS3 crosslinked (IL-12/BS3) were electrophoresed in parallel lanes as markers for the migration of the 75 kDa IL-12 heterodimer and for any oligomers of IL-12 that may form with the BS3 crosslinker. The molecular sizes indicated in the margins were estimated from standards run in parallel lanes. Exposure time was 7 days.

For Figure 10, Lymphoblasts ( $1 \times 10^6$  cells) were incubated for 2 hrs at room temperature with increasing concentrations of  $^{125}\text{I}$ -2-4E6 in the absence ( $^{\circ}$ ) or presence ( $\bullet$ ) of 25 nM unlabelled 2-4E6. Total ( $^{\circ}$ ) and non-specific ( $\bullet$ ) cell bound radioactivity were determined as described. Specific binding of  $^{125}\text{I}$ -2-4E6 ( $\nabla$ ) was calculated by subtracting non-specific binding from total binding. The right hand panel shows analysis of the binding data according to the method of Scatchard as determined by Ligand computer program with a single-site model.

For Figure 11, K6 cells ( $1 \times 10^6$  cells) were incubated for 2 hrs at room temperature with increasing concentrations of  $^{125}\text{I}$ -2-4E6 in the absence ( $\bullet$ ) or presence ( $\nabla$ ) of 25 nM unlabelled 2-4E6. Total ( $\bullet$ ) and non-specific ( $\nabla$ ) cell bound radioactivity were determined as described. Specific binding of  $^{125}\text{I}$ -2-4E6 ( $\nabla$ ) was calculated by subtracting non-specific binding from total binding. The right hand panel shows analysis of the binding data according to the method of Scatchard as determined by Ligand with a single-site model.

For Figure 12, The data are expressed as the amount of  $^{125}\text{I}$ -2-4E6 bound [CPM BOUND (Percent)] to the cells in the presence of the indicated concentrations of unlabelled antibody or IL-12 when compared with the total specific binding in the absence of unlabelled competitor.

For Figure 13, K6 cells ( $1 \times 10^6$  cells) were incubated for 2 hrs at room temperature with increasing concentrations of  $^{125}\text{I}$ -IL-12 in the absence ( $^{\circ}$ ) or presence ( $\bullet$ ) of 50 nM unlabelled IL-12. Total ( $^{\circ}$ ) and non-specific ( $\bullet$ ) cell bound radioactivity were determined as described. Specific binding of  $^{125}\text{I}$ -IL-12 ( $\nabla$ ) was calculated by subtracting non-specific binding from total binding. The right hand panel shows analysis of the binding data according to the method of Scatchard as determined by Ligand with a single-site model.

For Figure 14, K6 cells ( $1.5 \times 10^8$  cells/ml) were solubilized with 8 mM CHAPS extraction buffer and the cell extract (0.2 ml) was immunoprecipitated for 16 hrs at  $4^{\circ}\text{C}$  with mAb 2-4E6 immobilized on goat anti-mouse IgG coupled to agarose as described. Following this incubation, the beads were pelleted, washed and resuspended in IP buffer containing  $^{125}\text{I}$ -IL-12 at concentrations ranging from 7 pM to 7.5 nM. The IL-12R immobilized on the 2-4E6 coated beads was incubated with  $^{125}\text{I}$ -IL-12 for 2 hrs at RT and IL-12R bound radioactivity was determined in the presence of 50 nM unlabelled IL-12. The right hand panels show analysis of the binding data according to the method of Scatchard as determined by Ligand with a single-



site model.

For Figure 15, PHA-activated PBMC ( $1 \times 10^8$  cells/ml) were solubilized with 8 mM CHAPS extraction buffer and the cell extract (1 ml) was immunoprecipitated as described in Figure 13. Following this incubation, the beads were pelleted, washed and the bound proteins released by treatment with 0.1 M glycine pH 2.3. The released proteins were separated by non-reducing SDS/PAGE on 8% gels transferred to nitrocellulose membrane and probed with  $^{125}\text{I}$ -2-4E6 as described. The molecular sizes indicated in the margins were estimated from molecular weight standards (Amersham Prestained High Molecular Weight Standards) run in parallel lanes. Exposure time was 7 days.

Table 2

COMPARISON OF THE BINDING OF IL-12 AND 2-4C6 TO HUMAN CELLS EXPRESSING IL-12 RECEPTOR				
CELL TYPE	IL-12 BINDING <sup>1</sup>		2-4E6 BINDING <sup>2</sup>	
Human Cells	$K_D$ (nM)	Sites/cell	$K_D$ (nM)	Sites/cell
non-activated human PBMC <sup>3</sup>	none	detected	none	detected
PHA-PBMC (5-7 days)(3 sites)	0.018 0.084 1.800	312 501 1406	0.745	1472-2246
K6 cells (3 sites)	0.016 0.057 2.400	707 939 4036	0.489	3116-5259
Kit-225 (3 sites)	0.023 0.210 2.360	100 250 755	0.594	1950
YT cells (2 sites)	0.006 0.109	24 117	0.904	4522
RAJI cells	none	detectable	0.450	561
MRC-5	none	detectable	none	detectable
HL-60	none	detectable	none	detectable

<sup>1</sup> Steady state  $^{125}\text{I}$ -IL-12 binding assays. Apparent dissociation constant ( $K_D$ ) and binding sites per cell have been calculated by transformation of the data by the method of Scatchard.

<sup>2</sup> Steady state  $^{125}\text{I}$ -2-4E6 binding assays. Data transformed by the method of Scatchard.

<sup>3</sup> Human peripheral blood mononuclear cells (PBMC) were activated with PHA as described in the methods (PHA-PBMC).

#### Example 15

##### mAb 2-4E6 Binding To Human Recombinant IL-12R Expressed in COS Cells

The characteristics of the protein bound by mAb 2-4E6 fulfilled standard criterion for an IL-12R and therefore 2-4E6 was used in an expression cloning strategy to isolate a cDNA coding for the human IL-12R. A cDNA coding for the human IL-12R was isolated by this method. The IL-12R cDNA was engineered in a mammalian cell expression vector, transfected into COS-7 (ATCC CRL 1651) cells and the specificity for binding of  $^{125}\text{I}$ -IL-12 and  $^{125}\text{I}$ -2-4E6 was determined. Steady state binding of  $^{125}\text{I}$ -IL-12 to the rIL-12R expressing COS cells identifies a single binding site with an apparent affinity of 2-6 nM and approximately 150,000 sites/cell (Figure 4A). This low affinity IL-12 binding site corresponds to the low affinity site seen in the binding assays with human cells that naturally express IL-12R. The binding of  $^{125}\text{I}$ -2-4E6 to rIL-12R expressed in the COS cells is saturable and specific and identifies approximately 500,000 sites/cell (Figure 4B). COS cells transfected with an unrelated plasmid do not bind either  $^{125}\text{I}$ -IL-12 or  $^{125}\text{I}$ -2-4E6 (data not shown). These data demonstrated unequivocally that mAb 2-4E6 was specific for the low affinity component of the IL-12R.

For Figure 4A, COS cells were transfected with a plasmid expressing human rIL-12R as described. Three days later, transfected cells ( $1 \times 10^4$  cells) were incubated for 2 hrs at room temperature with increasing concentration of  $^{125}\text{I}$ -IL-12 in the absence (○) or presence (□) of 50 nM unlabelled IL-12. Total (○) and non-specific (□) cell bound radioactivity were determined as described. Specific binding of  $^{125}\text{I}$ -IL-12 (Δ) was calculated by subtracting non-specific binding from total binding. The right hand panel shows analysis of the binding data according to the method of Scatchard as determined by Ligand with a single-site model.

For Figure 4B, COS cells were transfected with a plasmid expressing human rIL-12R as described. Three days later, transfected cells ( $1 \times 10^4$  cells) were incubated for 2 hrs at room temperature with increasing concentrations of  $^{125}\text{I}$ -2-4E6 in the absence (○) or presence (□) of 50 nM unlabelled 2-4E6. Total (○) and non-specific (□) cell bound radioactivity were determined as described. Specific binding of  $^{125}\text{I}$ -2-4E6 (Δ) was calculated by subtracting non-specific binding from total binding. The right hand panel shows analysis of the binding data according to the method of Scatchard as determined by Ligand with a single-site model.

#### Example 16

##### Analysis of mAb 2-4E6 Binding to IL-12R Positive Human Cells by Fluorescence Activated Cell Sorting (FACS)

The expression level of IL-12R on human cells could be regulated depending on the activation state of the cells, the cell cycle or the type of environment from which the cells are isolated. Previous data had demonstrated that PHA activation of PBMC leads to a gradual rise in IL-12R expression, reaching a maximum at 3-4 days after activation and declining thereafter. Desai et al. (1992) J. Immunol., 148, 3125. To investigate the heterogeneity of IL-12R expression on PHA-activated PBMCs, Kit-225 and K6 cells, FACS analysis of IL-12R on these cells was determined with mAb 2-4E6 (Figure 16). The fluorescence intensity of binding of 2-4E6 was specific and indicated that these three cell types expressed approximately equal numbers of IL-12R. Interestingly, the FACS analysis indicated that the cell population was fairly homogenous and did not have one population expressing no or low numbers of IL-12R and a second population that expressed very high numbers of IL-12R.

For Figure 16, Day 4 PHA-activated lymphoblasts, Kit-225 and K6 cells were analyzed for IL-12R expressing cells by the indirect fluorescent antibody-labelling technique described. The figure depicts specific staining for IL-12R obtained in the presence of mAb 2-4E6 (IL-12R) and non-specific staining obtained in the presence of a control antibody specific for IL-1 receptor (anti-HuIL-1R), a control antibody specific for human IL-12 (4D6 + GART-PE CTRL) and the goat anti-mouse antibody conjugated with PE (GART-PE CTRL).

#### Example 17

##### Cell Culture

Peripheral blood mononuclear cells (PBMC) were isolated from blood collected from healthy donors. The blood was collected into heparinized syringes, diluted with an equal volume of Hank's balanced salt solution (HBSS) and layered over Ficoll-Hypaque. The tubes were spun at 2000 rpm for 20 minutes at room temperature. PBMC at the interface were collected and pelleted at 1500 rpm for 10 minutes through a 15 ml cushion of 20% sucrose in PBS. Pelleted PBMC were resuspended in tissue culture medium and washed twice in the same medium (RPMI 1640 plus 5 % serum). Finally, the cells were cultured at  $0.5 - 1 \times 10^6$  cells/ml in tissue culture medium plus 1 μg/ml PHA-P (Difco) for 3 days at 37 °C in a 5% CO<sub>2</sub> atmosphere. Cells were split 1:1 in culture medium plus 50 U/ml rhuIL-2 (Roche) to yield >95% T-cells. The next day, these cells were used for assessing their responsiveness to IL-12, for radioligand (IL-12) binding assays and in flow cytometry assays for the detection of IL-12 receptors.

Flow cytometric detection of IL-12 receptors on such 4 day activated PHA blasts was performed as follows: the cells were washed twice in PBS and resuspended at  $2 \times 10^6$  cells/ml in PBS plus 2% fetal calf serum and 0.1% sodium azide. All the subsequent steps were carried out at 4 °C.  $1 \times 10^6$  cells were incubated in 1 nM human IL-12 for 40 minutes. The cells were washed in FACS buffer and incubated with about 1 μg of biotinylated rat anti human p40 IL-12 subunit antibody 4D6 (Example 13) for 20 minutes. Cells were washed again and resuspended in 100 μl of a 5 μg/ml streptavidin-phycoerythrin conjugate (Fisher Biotech) for 15 minutes. The cells were then washed again before analysis on a FACSAN flow

cytometer (Becton Dickinson).

### Example 18

#### 5 Extraction and characterization of RNA

PHA activated cells as described above were harvested at day 2-3 and total RNA was extracted using GuanidiniIsothiocyanate/Phenol as described (P. Chomczynski and N. Sacchi (1987) Anal. Biochem., 162, 156). Poly A+ RNA was isolated from the total RNA by one batch adsorption to oligo dT latex beads as described (K. Kuribayashi et al (1988) Nucl. Acids Res. Symposium Series 19, 61). The mass yield of this  
10 purification was about 4%.

RNA blots were performed as follows. RNA was fractionated in 1.2 % agarose gels under denaturing conditions in the presence of 2.2M formaldehyde and subsequently transferred to nitrocellulose as described (Molecular Cloning, a Laboratory Manual, second edition, J. Sambrook, E.F. Fritsch, T. Maniatis, Cold Spring Harbour Laboratory Press 1989 (hereinafter "Molecular Cloning Manual")). The RNA blots were  
15 hybridized ( $7 \times 10^5$  cpm/ml, 30 ml) with labelled probe in 5 x SSC (1X SSC = 0.15 M NaCl-0.015 M NaCitrate) - 50 % formamide - 5 x Denhardtts solution (1 x Denhardtts = 0.02% polyvinylpyrrolidone, 0.02 % Ficoll 400, 0.02% bovine serum albumin fraction V) - 0.3 % SDS - 250 µg/ml denatured salmon sperm carrier DNA at 37°C overnight. The probe was generated by random-primer labelling gel-isolated insert  
20 from IL-12 receptor cDNA clone No. 5 as described in the above manual. The blots were first quickly rinsed at room temperature in 2 x SSC, then washed in 0.1 x SSC at 50°C for 30 minutes, dried and exposed to Kodak XAR film at -70°C for 3 days.

### Example 19

#### 25 cDNA Library

From the above polyA+ RNA, a cDNA library was established in the mammalian expression vector pEF-BOS as follows: 3 µg of polyA+ RNA were reverse transcribed into single stranded cDNA using  
30 RNaseH minus reverse transcriptase (GIBCO BRL Life Technologies Inc., P.O.Box 9418, Gaithersburg, MD 20898). The resulting mRNA-cDNA hybrids were converted into blunt ended double stranded cDNAs by established procedures (U.Gubler and A.Chua, in: Essential Molecular Biology Volume II, T.A. Brown, editor, pp. 39-56, IRL Press 1991). BstXI linkers (A. Aruffo and B. Seed, Proc. Natl. Acad. Sci. USA (1987) 84, 8573) were ligated to the resulting cDNAs and cDNA molecules > 800 base pairs (bp) were selected over a  
35 Sephacryl SF500 column. A Sephacryl SF 500 column (0.8 x 29 cm) was packed by gravity in 10 mM Tris-HCl pH 7.8 - 1 mM EDTA - 100 mM NaAcetate. BstXI linkered cDNA was applied to the column and 0.5 ml fractions were collected. A small aliquot of each fraction was run on a 1% agarose gel, the gel was dried down and the size distribution of the radioactive cDNA visualized by exposure of the gel to X-ray film. cDNA molecules larger than 800 bp were selected in this fashion, pooled, concentrated by ethanol  
40 precipitation and subsequently ligated to the cloning vector. The cloning vector was the plasmid pEF BOS (see reference supra) that had been cut with BstXI and purified over two consecutive gels. 300 ng of plasmid DNA were ligated to 30 ng of size selected cDNA from above in 60 µl of ligation buffer (50 mM Tris-HCl pH 7.8 - 10 mM MgCl<sub>2</sub> - 10 mM DTT - 1 mM rATP - 25 µg/ml bovine serum albumin) at 15°C overnight. The following day, the ligation reaction was extracted with phenol, 6 µg of mussel glycogen were  
45 added, and the nucleic acids were precipitated by ethanol. The precipitate was dissolved in water and the precipitation was repeated, followed by a wash with 80% ethanol. Finally, the pellet was dissolved in 6 µl of water and 1 µl aliquots were subsequently electroporated into *E. coli* strain DH-10B (BRL). By electroporating 5 parallel aliquots in this fashion, a library of about 10 million recombinants was generated for future use.

#### 50 Example 20

##### Screening for IL-12 Receptor cDNAs by Panning.

The basic principle of the panning method has been described in A. Aruffo and B. Seed, Proc. Natl. Acad. Sci. USA (1987) 84, 8573 as discussed below. Ten library aliquots each representing about 50,000 clones were plated on LB amp plates and were grown up overnight. The next day, the colonies from each pool were scraped off into a separate 50 ml aliquot of LB + amp and the cultures were grown for another

two hours before plasmid DNA was extracted using QIAGEN plasmid kits. The ten separate DNA pools were then transfected into COS cells, using the DEAE dextran technique (2 million COS cells/9 cm diameter plate and 2.5 µg DNA) (Molecular Cloning Manual). 2 to 3 days later, the COS cells were detached from the plates using 0.5 mM EDTA/0.02% Na Azide in PBS and a single cell suspension was prepared for each pool. The monoclonal anti IL-12 receptor antibody 2-4E6 as discussed above was subsequently bound to the cells in suspension (10 µg/ml in PBS-0.5mM EDTA-0.02% Na Azide-5% FCS, 1 hour, on ice). The cell suspension was then spun through a layer of 2% Ficoll in the above buffer (tabletop centrifuge, 1000 rpm, 4 minutes) to eliminate the excess unbound antibody and the cells were gently resuspended in the same buffer. The cells from one pool were subsequently added to one bacterial petri dish (9 cm diameter) that had been coated with polyclonal goat anti mouse IgG (20 µg/ml in 50 mM Tris-HCl pH 9.5, RT/OVERNIGHT (ON)) and blocked with 1% BSA in PBS (37°C/1 hour). COS cells were panned in this way for 2 hours at RT. Nonadhering cells were then gently washed off with PBS and the remaining adherent cells in the dishes lysed by the addition of 0.8 ml of Hirt lysis solution (0.6% SDS-10 mM EDTA). After transferring to Eppendorf tubes, the lysates were made 1 M NaCl, incubated ON at +4°C and then spun at 15,000 rpm for 10 minutes in the cold. The supernatants were extracted with phenol once, 12 µg of mussel glycogen was added and the DNA precipitated twice by adding 0.5 volumes of 7.5 M NH<sub>4</sub>OAc and 2.5 volumes of ethanol. The resulting DNA pellet was washed once with 80% ethanol, dried and taken up in 1 µl of distilled H<sub>2</sub>O. The entire prep was then electroporated into *E. coli* strain DH-10B and the resulting colonies grown up ON. This represents one panning cycle. The ten library aliquots were panned each one separately for a total of three cycles.

From the last cycle of each pool, DNA was again extracted and this time transfected into COS cells plated on plastic one-chamber microscopic slides (2 slides per pool). 2-3 days after transfection, to one of the slides was bound labelled human IL-12 (10<sup>6</sup> cpm/ml = 300 pM in RPMI 1640 plus 5% FCS for 2-3 hours at 4°C) and to the other slide labelled monoclonal Ab 2-4E6 (2 x 10<sup>6</sup> cpm/ml = 1 nM in RPMI 1640 plus 5% FCS for 1 hour at RT). The slides were washed in PBS, fixed for 40 seconds in a cold mixture of methanol:acetone (7:3) and air dried. The slides were subsequently dipped in Kodak photographic emulsion NTB2, air dried and exposed in a light-tight container for 2-4 days at 4°C. They were developed in Kodak D10 developer according to the manufacturer's instructions and evaluated under a light microscope using a 10 to 40 fold bright field magnification. One of the ten pools, number 5, showed a large number of positive cells both for IL-12 and 2-4E6 binding. *E. coli* clones from this 3 x panned pool were subsequently picked into a microtiterplate (3 clones per well for a total of 288 clones). Pools representing the 8 rows and 12 columns from this plate were grown up and their plasmid DNA extracted. These 20 preps were transfected separately into COS cells on 12 well plates (10<sup>5</sup> cells well, 4 wells per pool). 2-3 days after the transfection, labelled IL-12 was bound to the cells in two wells (total binding), whereas the other two wells per pool received labelled IL-12 and a 100 fold molar excess of cold IL-12 (= nonspecific binding). Wells were washed and the bound radioactivity eluted with 0.5 ml of 1% SDS and counted in a gamma counter. Two positive pools were identified in this manner, one representing column 1 and the other one representing row F from the microtiterplate. *E. coli* clones from well F1 must thus contain the IL-12 binding activity. Clones from that well were plated, and DNA from 10 single colonies was analyzed for plasmid insert size. 3 out of the 10 colonies showed an insert of about 2.1 kilobases in length, large enough to encode the IL-12 receptor. One of these clones was picked for further analysis.

#### Example 21

##### 45 Characterization of IL-12 Receptor cDNAs

IL-12 receptor clone No. 5 was picked as described above and the plasmid DNA isolated. Gel isolated insert was sequenced on both strands using the ABI automated DNA sequencer in conjunction with a thermostable DNA polymerase and dye-labelled dideoxynucleotides as terminators.

50 Sequence alignments were run using the ALIGN program (M. O. Dayhoff et al. (1983) Meth. Enzymol. 91, 524) with the mutation data matrix, a break penalty of 6 and 100 random runs.

Cloned IL-12 receptor cDNAs were expressed in COS cells using either the DEAE dextran transfection or electroporation techniques (Molecular Cloning Manual). Binding assays with labelled IL-12 or labelled 2-4E6 antibody were run as described herein above under anti human IL-12 receptor antibody. The binding data were analyzed and K<sub>d</sub> values were calculated according to Scatchard, using the Ligand program discussed herein above under anti human IL-12 receptor antibody. *In vivo* labelling (6 hours) of COS cells (3 x 10<sup>5</sup> cells per 35 mm diameter tissue culture dish) with <sup>35</sup>S Cysteine was performed 3 days after transfection as described (Molecular Cloning Manual). Cells were washed in PBS and lysed in CHAPS lysis

buffer (10 mM CHAPS - 300 mM NaCl - 50 mM Tris-HCl pH 7.4 - 2 mg/ml Iodoacetamide - 0.17 mg/ml PMSF), precleared by incubation with protein G Sepharose beads (50  $\mu$ l packed beads per ml, Genex) and normal mouse serum (25 % final concentration) at 4°C overnight. The beads were spun out and labelled IL-12 receptor was specifically immunoprecipitated from the cleared lysates by adding 5  $\mu$ g of 2-4E6 antibody per ml of sample. The antibody was diluted in PBS containing 1% bovine serum albumin and had been loaded onto 50  $\mu$ l of packed beads for 2 - 3 hours at 4°C. Immunoprecipitation took place overnight at 4°C. The next day, the beads were washed 3-4 times in CHAPS lysis buffer before analysis on SDS-polyacrylamide gels as described (Molecular Cloning Manual).

## Example 22

### Lymphocyte Proliferation Assay

Lymphocyte proliferation assays to assess the ability of rat antisera to block cytokine-induced proliferation were performed as previously described (M.K. Gately, et al. (1992) Current Protocols in Immunology, vol. 1., J. E. Coligan, et al., eds., John Wiley & Sons, New York, NY, p. 6.16.1) with the following modifications. Aliquots of human PHA-activated PBMC ( $2 \times 10^4$  per well) and of diluted rat sera were mixed in the wells of a 96-well plate and incubated at 37°C for 30 min. The cytokines (IL-12, IL-2 or IL-7) were then added to the wells, and the cultures were incubated for 48 h at 37°C. Following this,  $^3$ H-TdR was added to each well, and the cultures were harvested after an additional 7 h at 37°C. All values are the means of triplicates.

## Example 23

### Flow Cytometry

The titers of anti-COS cell antibodies in the various rat antisera were assessed by flow cytometry as follows. Untransfected COS cells ( $10^6$  cells/0.1 ml of Dulbecco's PBS containing 2% heat-inactivated FCS and 0.1 % sodium azide) were preincubated with 400  $\mu$ g/ml normal rat IgG (Sigma, St. Louis, MO) for 15 min. on ice, and then with the indicated amount of rat serum for 30 min. on ice. The cells were washed and further incubated with 2  $\mu$ g/ml FITC-conjugated F(ab')<sub>2</sub> mouse anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 30 min. on ice. The cells were again washed and then analyzed by flow cytometry using a FACScan (Becton-Dickinson, Mountain View, CA).

## Example 24

### Inhibition of IL-12-induced Lymphoblast Proliferation by Anti-IL-12R Antiserum

To determine whether the cloned IL-12R subunit plays an essential role in an IL-12-induced biologic response, we examined whether antiserum to the cloned IL-12R subunit could inhibit IL-12-induced proliferation of PHA-activated PBMC. This antiserum was produced by immunizing rats with 2-4E6-transfected COS cells and thus contained anti-COS cell antibodies as well as antibodies to the (putative) IL-12R subunit. For use as a control, we screened several other available rat antisera which had been prepared previously by immunization of rats with COS cells expressing proteins unrelated to the IL-12R. One such antiserum had been raised against COS cells transfected with human type II IL-1R and displayed a titer of anti-COS cell antibodies virtually identical to that of the anti-IL-12R antiserum (Figure 18A). We then compared the effects of the anti-IL-12R antiserum, the anti-IL-1R antiserum, and preimmune serum (from the rat used to prepare the anti-IL-12R) on lymphoblast proliferation induced by IL-12, IL-2, or IL-7. The concentrations of IL-12, IL-2, and IL-7 were 0.25 ng/ml, 1.25 ng/ml, and 0.4 ng/ml, respectively. These concentrations were chosen because they resulted in similar levels of  $^3$ H-TdR incorporation and were on the steep portion of the dose-response curves. In this experiment, the maximum levels of  $^3$ H-TdR incorporation in the presence of saturating amounts of cytokine were 38,820, 111,303, and 89,541 cpm for IL-12, IL-2, and IL-7, respectively. The level of  $^3$ H-TdR incorporation in the absence of any added cytokine is indicated by the horizontal dotted line. Two experiments were performed with essentially identical results, and one of these is illustrated in Figures 18B-D. The anti-IL-12R antiserum caused dose-dependent inhibition of IL-12-induced lymphoblast proliferation but had no effect on proliferation induced by IL-2 or IL-7. In contrast, neither the preimmune serum nor the anti-IL-1R antiserum inhibited lymphoblast proliferation induced by any of the three cytokines tested. These results strongly suggest that the cloned IL-12R subunit plays an

essential role in mediating IL-12-induced proliferation of PHA-activated PBMC.

#### Example 25

##### 5 Sequence Analysis of IL-12 Receptor cDNA Clones

The DNA sequence for the IL-12 receptor cDNA insert from clone No. 5 is shown in Figure 1. The deduced amino acid sequence for the encoded receptor protein is shown in Figure 2. The IL-12 receptor protein is thus composed of 662 amino acids, and has the following features: N-terminal signal peptide, 10 extracellular domain, transmembrane domain and cytoplasmic tail. The classical hydrophobic N-terminal signal peptide is predicted to be 20-24 amino acids in length. Signal peptide cleavage has been shown to occur mostly after the amino acids Ala, Ser, Gly, Cys, Thr, Gln (G. von Heijne, Nucl. Acids Res. (1986) 14, 4683). For the IL-12 receptor, the cleavage could thus take place after Gln20, Ala23 or Cys24 in the sequence shown in Figure 2, leaving a mature protein of 638 amino acids (calculated molecular weight = 15 70,426) based as cleavage at Cys24. The extracellular domain of the receptor is predicted to encompass the region from the C-terminus of the signal peptide to amino acid No. 540 in the sequence shown in Figure 2. Hydrophobicity analysis shows the area from amino acid No. 541 to 571 to be hydrophobic, as would be expected for a transmembrane anchor region. Charged transfer stop residues can be found at the N- as well as the C-terminus of this predicted transmembrane area. The extracellular domain of the receptor is thus 20 516 amino acids long and contains all the 6 predicted N-linked glycosylation sites. The cytoplasmic portion is 91 amino acids long (amino acid residue nos. 572 to 662) and contains 3 potential phosphorylation sites (S/TXXD/E) for casein kinase II.

The cDNA library was rescreened using the insert from clone No. 5 as the probe, and a second independent cDNA was isolated (clone No. 17). This cDNA contained an additional 202 bp of 3' 25 untranslated region. The amino acid sequence (SEQ ID NO:3) deduced from this clone for the IL-12 receptor protein was almost completely identical to the sequence shown in Figure 2 (SEQ ID NO:2); however, a 13 bp deletion in the cDNA right before the stop codon changes the reading frame at the very C-terminus of the receptor and also gives rise to a protein that is 2 amino acids shorter (SEQ ID NO:3). Cycle sampling PCR was performed on uncloned cDNA using a pair of primers spanning the region that is 30 expected to differ between the mRNAs representing clones 5 and 17. This analysis demonstrated that both transcripts coding for these two membrane-bound variants of the receptor subunit are present in the mRNA population isolated from PHA-activated PBMC at about equal levels (data not shown). The two transcripts are likely to arise from an alternate splicing event.

Further analysis of the amino acid sequence of the IL-12 receptor shows it to be a member of the 35 cytokine receptor superfamily, by virtue of the sequence motifs [Cys52 --- Cys62SW] and [W222SKWS]. Comparing the IL-12 receptor sequence to all the members of the superfamily by running the ALIGN program shows that the IL-12 receptor has the highest homology to human gp130.

Sequence analysis of the IL-12 receptor extracellular domain demonstrated the presence of the hemopoietin receptor hallmark features: two pairs of conserved cysteine-residues and the WSXWS motif 40 Further comparisons to the hemopoietin receptor superfamily showed the newly isolated IL-12 receptor component to be highly related to a subgroup of family members composed of gp130, G-CSF-receptor and LIF-receptor (Figure 3); align scores were 12.37 (IL-12R/gp130), 7.35 (IL-12R/G-CSF-R) and 6.31 (IL-12R/LIF-Rbeta). Similarities between the IL-12 receptor component and these three proteins extend beyond the hemopoietin receptor domain and include the area from the WSXWS motif to the transmembrane region 45 (Figure 3). The extracellular portion of gp130 (M. Hibi et al. (1990) Cell, 63, 1149) was shown i) to contain the hemopoietin receptor superfamily domain and ii) to be also composed of 6 type III fibronectin repeats about 90 amino acids long (A.R. Kornblihtt, et al. (1985) EMBO J., 4, 1755; L. Patthy (1990) Cell 61, 13). Similarly, the extracellular domain of the IL-12 receptor can be subdivided into five such fibronectin type III repeats (residues 43-133, 143-236, 237-337, 338-444 and 445-540). The IL-12 receptor extracellular domain 50 lacks the N-terminal Ig domain found in gp130 and therefore only accommodates 5 fibronectin type III repeats. Further sequence similarities between the IL-12 receptor, gp130, the G-CSF-receptor and the LIF-receptor can be found in the cytoplasmic regions (Figure 3). A PXXPP motif within a box of 8 amino acids conserved between a number of different superfamily members and a second, 12 amino acid long conserved box were found to be important for signal transduction mediated by gp130 (M. Murakami et al. 55 (1991) Proc. Natl. Acad. Sci. (USA), 88, 11349). Both those motifs are also found in conserved areas of the cytoplasmic part of the IL-12 receptor sequence (amino acid residues 577-584 and amino acid residues 618-629).

Example 26Analysis of IL-12 Receptor mRNA Expression

RNA blots were performed using polyA<sup>+</sup> RNA from cells known to respond to IL-12: PHA-stimulated PBMC and the CD4<sup>+</sup> T-cell line Kit225. Two RNA transcripts about 3 Kb and 2.3 Kb in size are apparent when blots are probed with the full-length cDNA (Figure 6, lanes 1-3). Both RNAs are induced from undetectable or very low levels when PBMC are activated by PHA for 3 days (compare lanes 1 and 2); Kit225 cells express both transcripts constitutively (lane 3). Analysis by phosphorimager shows the level of the larger RNA to be about 3 to 5 fold higher than the level of the smaller RNA. Surprisingly, the smaller RNA does not hybridize to a probe derived from the cytoplasmic domain (lanes 4-6). This finding could indicate the presence of an RNA encoding i) a soluble IL-12 receptor protein, ii) a membrane bound IL-12 receptor devoid of a cytoplasmic region altogether or iii) an IL-12 receptor with a cytoplasmic sequence that is completely different from the ones present in clones 5 and 17. Elucidation of this question will have to await the isolation of a cDNA clone derived from the smaller RNA transcript.

Example 27Characterization of Recombinant IL-12 Receptor

IL-12 receptor cDNA (clone number 5) (SEQ ID NO:1) was electroporated into COS cells and equilibrium binding of labelled human and murine IL-12 to the cells was performed and analyzed as described (R. Chizzonite, et al., 1992, J. Immunol., 148, 3117). Results are shown in Figure 19. Human and murine IL-12 bind to recombinant IL-12 receptor (SEQ ID NO:2) with a single affinity ( $K_D$ ) of  $3.4 \pm 1.3$  nM ( $n=7$ ) and  $2.1 \pm 0.5$  nM ( $n=4$ ), respectively, which corresponds to the low affinity component of the functional IL-12 receptor on PHA-activated PBMC. After transformation by the method of Scatchard, the equilibrium binding data was best described by a single receptor site model as determined by the LIGAND program. The site numbers indicated in Figure 19 are calculated assuming that all cells are expressing receptors. IL-12 receptor protein expressed by clone number 17 (SEQ ID NO:3) gave similar results in these binding assays. SEQ ID NO:3 would also have the same regions as SEQ ID NO:2.

Metabolic labelling and immunoprecipitation of the IL-12 receptor subunit expressed in COS cells indicated its size to be about 100 KDa as determined by gel analysis under reducing conditions (Figure 5). To analyze the size of the receptor at the cell surface, affinity crosslinking studies were performed. Unless otherwise stated, characterization of the IL-12 receptor protein was done on SEQ ID NO:2. Crosslinking of 0.2 nM <sup>125</sup>I-labelled IL-12 to either transfected COS cells, PHA-activated PBMC or K6 cells gave rise to complexes that migrate at >200 KDa (Figure 17, lanes 1,3 and 4; arrow indicates uncrosslinked IL-12). Crosslinking at 2 nM <sup>125</sup>I-IL-12 (a concentration equivalent to the  $K_D$ ) gave identical results (not shown). The size of a complex composed of one receptor subunit and one IL-12 ligand is expected to be about 175 KDa. However, Figure 17 shows that the 175 KDa complex is present only at very low levels, if at all. Since the 150 kDa Ig and the 200 KDa markers are not separated on the gel system used, the 175 KDa IL-12/IL-12 receptor complex is expected to comigrate with them. For comparison, lane 2 shows transfected COS cells crosslinked to labelled 2-4E6 antibody (arrowhead = uncrosslinked 2-4E6). Crosslinking labelled IL-12 to i) cells that do not bind IL-12 (e.g. Raji cells), ii) mock-transfected COS cells or iii) transfected COS cells in the presence of an excess of cold IL-12 did not yield any products (not shown). For Figure 17, labelled IL-12 (0.2 nM) was bound and crosslinked with BS3 (0.4 mM) to transfected COS cells (lane 1), PHA-activated PBMC (lane 3) or K6 cells (lane 4). Labelled 2-4E6 antibody (0.5 nM) was bound and crosslinked with BS3 (0.4 mM) to transfected COS cells (lane 2). Anti-IL-12 receptor antibody 2-4E6 (lanes 5,7), anti-IL12 antibody 4D6 (lanes 9,11) and control antibody (lanes 6,8,10,12,) were used. Antibody 2-4E6 (lanes 13,15) and control antibody (lanes 14,16) were used.

Since crosslinking of labelled IL-12 to IL-12 receptor gave rise to products that are larger than what is expected for a complex of one receptor and one IL-12 ligand but whose size is difficult to estimate, cell surface labelling and immunoprecipitation experiments of transfected COS cells were performed. Samples were analyzed under reducing and nonreducing conditions (Figure 17, lanes 5-12). The results can be summarized as follows: i) transfected COS cells express the IL-12 receptor subunit both as monomers and as a second, larger product that could be dimers or oligomers. Both these products are present at about equal levels (lane 5); ii) the dimerization/oligomerization does not depend on IL-12 binding. If IL-12 is prebound to the cells, the resulting banding pattern does not change (not shown); and iii) The dimers/oligomers can be converted to the monomers by reduction and must therefore be disulfide-bonded



(lane 7). The data from the crosslinking and surface labelling experiments thus suggested that only the dimeric/oligomeric receptor subunit form binds IL-12 with the 3 nM affinity measured on transfected COS cells. This possibility was further investigated as follows. Complexes produced by binding unlabelled IL-12 to <sup>125</sup>I-surface labelled COS cells and crosslinking with a cleavable crosslinker were immunoprecipitated by an anti-IL-12 antibody and analyzed under non-reducing and reducing conditions (Figure 17, lanes 9-12). The anti-IL-12 antibody only precipitated a complex corresponding to IL-12 bound to the dimer/oligomer but not the monomer of the IL-12 receptor subunit (lane 9). Analysis of this complex under reducing conditions identified a labelled protein that co-migrated with the IL-12 receptor monomer (lane 11). Experiments with a murine CTLL cell transfectant stably expressing the IL-12 receptor subunit lend further support to our findings. These cells express about 3000 to 5000 receptor subunits per cell, as measured by 2-4E6 antibody binding; however, the cells bind IL-12 very inefficiently, with an estimated K<sub>d</sub> of 50 nM or greater (not shown). The results from surface labelling and immunoprecipitation experiments with the CTLL transfectants clearly indicate that they only express IL-12 receptor subunit monomers (Figure 17, lanes 13-16). Taken together, the data support the hypothesis that only the receptor subunit dimers/oligomers bind IL-12 with the low affinity (3 nM) measured on transfected COS cells.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: F. HOFFMANN-LA ROCHE AG  
 (B) STREET: Grenzacherstrasse 124  
 (C) CITY: Basle  
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 (F) POSTAL CODE (ZIP): CH-4002  
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 (I) TELEX: 962292/965542 hlr ch

(ii) TITLE OF INVENTION: INTERLEUKIN-12 RECEPTOR

(iii) NUMBER OF SEQUENCES: 3

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
 (B) COMPUTER: Apple Macintosh  
 (C) OPERATING SYSTEM: System 7.1 (Mac)  
 (D) SOFTWARE: Microsoft Word 5.0

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2104 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens  
 (G) CELL TYPE: human T-cells

## (vii) IMMEDIATE SOURCE:

(A) LIBRARY: library 3 day PHA/pEF-BOS  
 (B) CLONE: human interleukin-12 receptor clone #5

## (ix) FEATURE:

(A) NAME/KEY: CDS  
 (B) LOCATION: 65..2050

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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EP 0 638 644 A1

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	Glu	Val	Thr	Leu	Gln	Leu	Tyr	Asn	Ser	Val	Lys	Tyr	Glu	Pro	Pro	Leu	
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	Gly	Asp	Ile	Lys	Val	Ser	Lys	Leu	Ala	Gly	Gln	Leu	Arg	Met	Glu	Trp	
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	ACA	CCC	AGC	AGC	CCA	TGG	AAG	TTG	GGC	GAC	TGC	GGA	CCT	CAG	GAT	GAT	637
	Thr	Pro	Ser	Ser	Pro	Trp	Lys	Leu	Gly	Asp	Cys	Gly	Pro	Gln	Asp	Asp	
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	Asp	Thr	Glu	Ser	Cys	Leu	Cys	Pro	Leu	Glu	Met	Asn	Val	Ala	Gln	Glu	
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40	Phe	Gln	Leu	Arg	Arg	Arg	Gln	Leu	Gly	Ser	Gln	Gly	Ser	Ser	Trp	Ser	
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25	AAC GGG ACC ACC ATG TAT TGG CCA GCC CGG GCT CAG AGC ATG ACG TAT	1165
	Asn Gly Thr Thr Met Tyr Trp Pro Ala Arg Ala Gln Ser Met Thr Tyr	
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30	TGC ATT GAA TGG CAG CCT GTG GGC CAG GAC GGG GGC CTT GCC ACC TGC	1213
	Cys Ile Glu Trp Gln Pro Val Gly Gln Asp Gly Gly Leu Ala Thr Cys	
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	Ser Trp Ser Arg Glu Ser Gly Ala Met Gly Gln Glu Lys Cys Tyr Tyr	
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## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 662 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (ix) FEATURE:

- (A) NAME/KEY: Region
- (B) LOCATION: 1..23
- (D) OTHER INFORMATION: /note= "N-terminal signal peptide (1..20 or 23 or 24)"

## (ix) FEATURE:

- (A) NAME/KEY: Region
- (B) LOCATION: 541..570
- (D) OTHER INFORMATION: /note= "transmembrane region"

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    (D) OTHER INFORMATION: /note= "cytoplasmic tail region"

5

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15

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    (A) NAME/KEY: Region  
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25

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30

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35

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40

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45

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    (B) LOCATION: 442..444  
    (D) OTHER INFORMATION: /note= "N-linked glycosylation  
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50

55

## (ix) FEATURE:

(A) NAME/KEY: Region

(B) LOCATION: 456..458

(D) OTHER INFORMATION: /note= "N-linked glycosylation site"

## (ix) FEATURE:

(A) NAME/KEY: Region

(B) LOCATION: 24..540

(D) OTHER INFORMATION: /note= "Extracellular region"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Pro Leu Val Thr Trp Val Val Pro Leu Leu Phe Leu Phe Leu  
1 5 10 15

Leu Ser Arg Gln Gly Ala Ala Cys Arg Thr Ser Glu Cys Cys Phe Gln  
20 25 30

Asp Pro Pro Tyr Pro Asp Ala Asp Ser Gly Ser Ala Ser Gly Pro Arg  
35 40 45

Asp Leu Arg Cys Tyr Arg Ile Ser Ser Asp Arg Tyr Glu Cys Ser Trp  
50 55 60

Gln Tyr Glu Gly Pro Thr Ala Gly Val Ser His Phe Leu Arg Cys Cys  
65 70 75 80

Leu Ser Ser Gly Arg Cys Cys Tyr Phe Ala Ala Gly Ser Ala Thr Arg  
85 90 95

Leu Gln Phe Ser Asp Gln Ala Gly Val Ser Val Leu Tyr Thr Val Thr  
100 105 110

Leu Trp Val Glu Ser Trp Ala Arg Asn Gln Thr Glu Lys Ser Pro Glu  
115 120 125

Val Thr Leu Gln Leu Tyr Asn Ser Val Lys Tyr Glu Pro Pro Leu Gly  
130 135 140

Asp Ile Lys Val Ser Lys Leu Ala Gly Gln Leu Arg Met Glu Trp Glu  
145 150 155 160

Thr Pro Asp Asn Gln Val Gly Ala Glu Val Gln Phe Arg His Arg Thr  
165 170 175

Pro Ser Ser Pro Trp Lys Leu Gly Asp Cys Gly Pro Gln Asp Asp Asp  
180 185 190

Thr Glu Ser Cys Leu Cys Pro Leu Glu Met Asn Val Ala Gln Glu Phe  
195 200 205

Gln Leu Arg Arg Arg Gln Leu Gly Ser Gln Gly Ser Ser Trp Ser Lys  
210 215 220

Trp Ser Ser Pro Val Cys Val Pro Pro Glu Asn Pro Pro Gln Pro Gln  
225 230 235 240

Val Arg Phe Ser Val Glu Gln Leu Gly Gln Asp Gly Arg Arg Arg Leu  
245 250 255



EP 0 638 644 A1

Thr Leu Lys Glu Gln Pro Thr Gln Leu Glu Leu Pro Glu Gly Cys Gln  
260 265 270

5 Gly Leu Ala Pro Gly Thr Glu Val Thr Tyr Arg Leu Gln Leu His Met  
275 280 285

Leu Ser Cys Pro Cys Lys Ala Lys Ala Thr Arg Thr Leu His Leu Gly  
290 295 300

10 Lys Met Pro Tyr Leu Ser Gly Ala Ala Tyr Asn Val Ala Val Ile Ser  
305 310 315 320

Ser Asn Gln Phe Gly Pro Gly Leu Asn Gln Thr Trp His Ile Pro Ala  
325 330 335

15 Asp Thr His Thr Glu Pro Val Ala Leu Asn Ile Ser Val Gly Thr Asn  
340 345 350

Gly Thr Thr Met Tyr Trp Pro Ala Arg Ala Gln Ser Met Thr Tyr Cys  
355 360 365

20 Ile Glu Trp Gln Pro Val Gly Gln Asp Gly Gly Leu Ala Thr Cys Ser  
370 375 380

Leu Thr Ala Pro Gln Asp Pro Asp Pro Ala Gly Met Ala Thr Tyr Ser  
385 390 395 400

25 Trp Ser Arg Glu Ser Gly Ala Met Gly Gln Glu Lys Cys Tyr Tyr Ile  
405 410 415

Thr Ile Phe Ala Ser Ala His Pro Glu Lys Leu Thr Leu Trp Ser Thr  
420 425 430

30 Val Leu Ser Thr Tyr His Phe Gly Gly Asn Ala Ser Ala Ala Gly Thr  
435 440 445

Pro His His Val Ser Val Lys Asn His Ser Leu Asp Ser Val Ser Val  
450 455 460

35 Asp Trp Ala Pro Ser Leu Leu Ser Thr Cys Pro Gly Val Leu Lys Glu  
465 470 475 480

Tyr Val Val Arg Cys Arg Asp Glu Asp Ser Lys Gln Val Ser Glu His  
485 490 495

40 Pro Val Gln Pro Thr Glu Thr Gln Val Thr Leu Ser Gly Leu Arg Ala  
500 505 510

Gly Val Ala Tyr Thr Val Gln Val Arg Ala Asp Thr Ala Trp Leu Arg  
515 520 525

Gly Val Trp Ser Gln Pro Gln Arg Phe Ser Ile Glu Val Gln Val Ser  
530 535 540

45 Asp Trp Leu Ile Phe Phe Ala Ser Leu Gly Ser Phe Leu Ser Ile Leu  
545 550 555 560

Leu Val Gly Val Leu Gly Tyr Leu Gly Leu Asn Arg Ala Ala Arg His  
565 570 575

50 Leu Cys Pro Pro Leu Pro Thr Pro Cys Ala Ser Ser Ala Ile Glu Phe  
580 585 590

55

Pro Gly Gly Lys Glu Thr Trp Gln Trp Ile Asn Pro Val Asp Phe Gln  
595 600 605

Glu Glu Ala Ser Leu Gln Glu Ala Leu Val Val Glu Met Ser Trp Asp  
610 615 620

5 Lys Gly Glu Arg Thr Glu Pro Leu Glu Lys Thr Glu Leu Pro Glu Gly  
625 630 635 640

Ala Pro Glu Leu Ala Leu Asp Thr Glu Leu Ser Leu Glu Asp Gly Asp  
645 650 655

10 Arg Cys Lys Ala Lys Met  
660

## (2) INFORMATION FOR SEQ ID NO:3:

15

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 660 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

25

Met Glu Pro Leu Val Thr Trp Val Val Pro Leu Leu Phe Leu Phe Leu  
1 5 10 15

30

Leu Ser Arg Gln Gly Ala Ala Cys Arg Thr Ser Glu Cys Cys Phe Gln  
20 25 30

Asp Pro Pro Tyr Pro Asp Ala Asp Ser Gly Ser Ala Ser Gly Pro Arg  
35 40 45

Asp Leu Arg Cys Tyr Arg Ile Ser Ser Asp Arg Tyr Glu Cys Ser Trp  
50 55 60

Gln Tyr Glu Gly Pro Thr Ala Gly Val Ser His Phe Leu Arg Cys Cys  
65 70 75 80

35

Leu Ser Ser Gly Arg Cys Cys Tyr Phe Ala Ala Gly Ser Ala Thr Arg  
85 90 95

Leu Gln Phe Ser Asp Gln Ala Gly Val Ser Val Leu Tyr Thr Val Thr  
100 105 110

40

Leu Trp Val Glu Ser Trp Ala Arg Asn Gln Thr Glu Lys Ser Pro Glu  
115 120 125

Val Thr Leu Gln Leu Tyr Asn Ser Val Lys Tyr Glu Pro Pro Leu Gly  
130 135 140

45

Asp Ile Lys Val Ser Lys Leu Ala Gly Gln Leu Arg Met Glu Trp Glu  
145 150 155 160

Thr Pro Asp Asn Gln Val Gly Ala Glu Val Gln Phe Arg His Arg Thr  
165 170 175

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Pro Ser Ser Pro Trp Lys Leu Gly Asp Cys Gly Pro Gln Asp Asp Asp  
180 185 190

55

	Thr	Glu	Ser	Cys	Leu	Cys	Pro	Leu	Glu	Met	Asn	Val	Ala	Gln	Glu	Phe	
			195					200					205				
5	Gln	Leu	Arg	Arg	Arg	Gln	Leu	Gly	Ser	Gln	Gly	Ser	Ser	Trp	Ser	Lys	
		210					215					220					
	Trp	Ser	Ser	Pro	Val	Cys	Val	Pro	Pro	Glu	Asn	Pro	Pro	Gln	Pro	Gln	
	225					230					235					240	
10	Val	Arg	Phe	Ser	Val	Glu	Gln	Leu	Gly	Gln	Asp	Gly	Arg	Arg	Arg	Leu	
					245					250					255		
	Thr	Leu	Lys	Glu	Gln	Pro	Thr	Gln	Leu	Glu	Leu	Pro	Glu	Gly	Cys	Gln	
				260					265					270			
15	Gly	Leu	Ala	Pro	Gly	Thr	Glu	Val	Thr	Tyr	Arg	Leu	Gln	Leu	His	Met	
			275					280					285				
	Leu	Ser	Cys	Pro	Cys	Lys	Ala	Lys	Ala	Thr	Arg	Thr	Leu	His	Leu	Gly	
		290					295					300					
20	Lys	Met	Pro	Tyr	Leu	Ser	Gly	Ala	Ala	Tyr	Asn	Val	Ala	Val	Ile	Ser	
	305					310					315					320	
	Ser	Asn	Gln	Phe	Gly	Pro	Gly	Leu	Asn	Gln	Thr	Trp	His	Ile	Pro	Ala	
				325						330					335		
25	Asp	Thr	His	Thr	Glu	Pro	Val	Ala	Leu	Asn	Ile	Ser	Val	Gly	Thr	Asn	
				340					345					350			
	Gly	Thr	Thr	Met	Tyr	Trp	Pro	Ala	Arg	Ala	Gln	Ser	Met	Thr	Tyr	Cys	
			355					360					365				
30	Ile	Glu	Trp	Gln	Pro	Val	Gly	Gln	Asp	Gly	Gly	Leu	Ala	Thr	Cys	Ser	
		370					375					380					
	Leu	Thr	Ala	Pro	Gln	Asp	Pro	Asp	Pro	Ala	Gly	Met	Ala	Thr	Tyr	Ser	
	385					390					395					400	
35	Trp	Ser	Arg	Glu	Ser	Gly	Ala	Met	Gly	Gln	Glu	Lys	Cys	Tyr	Tyr	Ile	
				405						410					415		
	Thr	Ile	Phe	Ala	Ser	Ala	His	Pro	Glu	Lys	Leu	Thr	Leu	Trp	Ser	Thr	
				420					425					430			
40	Val	Leu	Ser	Thr	Tyr	His	Phe	Gly	Gly	Asn	Ala	Ser	Ala	Ala	Gly	Thr	
			435					440					445				
	Pro	His	His	Val	Ser	Val	Lys	Asn	His	Ser	Leu	Asp	Ser	Val	Ser	Val	
		450					455					460					
45	Asp	Trp	Ala	Pro	Ser	Leu	Leu	Ser	Thr	Cys	Pro	Gly	Val	Leu	Lys	Glu	
	465					470					475					480	
	Tyr	Val	Val	Arg	Cys	Arg	Asp	Glu	Asp	Ser	Lys	Gln	Val	Ser	Glu	His	
				485						490					495		
50	Pro	Val	Gln	Pro	Thr	Glu	Thr	Gln	Val	Thr	Leu	Ser	Gly	Leu	Arg	Ala	
				500					505					510			
	Gly	Val	Ala	Tyr	Thr	Val	Gln	Val	Arg	Ala	Asp	Thr	Ala	Trp	Leu	Arg	
			515					520					525				

Gly Val Trp Ser Gln Pro Gln Arg Phe Ser Ile Glu Val Gln Val Ser  
 530 535 540  
 5 Asp Trp Leu Ile Phe Phe Ala Ser Leu Gly Ser Phe Leu Ser Ile Leu  
 545 550 555 560  
 Leu Val Gly Val Leu Gly Tyr Leu Gly Leu Asn Arg Ala Ala Arg His  
 565 570 575  
 10 Leu Cys Pro Pro Leu Pro Thr Pro Cys Ala Ser Ser Ala Ile Glu Phe  
 580 585 590  
 Pro Gly Gly Lys Glu Thr Trp Gln Trp Ile Asn Pro Val Asp Phe Gln  
 595 600 605  
 15 Glu Glu Ala Ser Leu Gln Glu Ala Leu Val Val Glu Met Ser Trp Asp  
 610 615 620  
 Lys Gly Glu Arg Thr Glu Pro Leu Glu Lys Thr Glu Leu Pro Glu Gly  
 625 630 635 640  
 20 Ala Pro Glu Leu Ala Leu Asp Thr Glu Leu Ser Leu Glu Asp Gly Asp  
 645 650 655  
 Arg Cys Asp Arg  
 660  
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### Claims

- 30 1. A DNA molecule coding for a low affinity Interleukin-12 receptor or a functional derivative thereof which binds specifically to Interleukin-12.
2. A DNA molecule of claim 1 coding for a human low affinity Interleukin-12 receptor or a functional derivative thereof which binds specifically to Interleukin-12.
- 35 3. A DNA molecule of claims 1-2 having the sequence SEQ ID NO:1 or a degenerate variant thereof.
4. A DNA molecule of claims 1-3 which codes for the amino acid sequence SEQ ID NO:2 or SEQ ID NO:3 or portions thereof.
- 40 5. A DNA molecule of claims 1-4 which codes for a soluble, truncated Interleukin-12 receptor protein.
6. A vector comprising a DNA molecule as claimed in any of claims 1-5.
- 45 7. A host cell which has been transformed by a vector of claim 6.
8. A low affinity Interleukin-12 receptor protein or a functional derivative thereof which binds specifically to Interleukin-12.
- 50 9. A low affinity Interleukin-12 receptor protein encoded by a DNA molecule of claims 1-5.
10. A low affinity Interleukin-12 receptor protein of claims 8-9 wherein the Interleukin receptor has a  $K_D$  of about 2 to about 10 nM, preferably a  $K_D$  of about 2 to about 5 nM.
- 55 11. A low affinity Interleukin-12 receptor protein of claims 8-10 having an apparent molecular weight of about 100 kDa determined by gel electrophoresis under reducing conditions.

12. A low affinity Interleukin-12 receptor protein of claims 8-11 which is a dimer or oligomer of the 100 kD receptor subunit.
- 5 13. A low affinity Interleukin-12 receptor protein of claims 8-12 which corresponds to the low affinity component of the functional Interleukin-12 receptor on PHA-activated PBMC.
14. An immunoglobulin capable of binding selectively to an Interleukin-12 receptor protein as defined in any one of claims 8-13.
- 10 15. An immunoglobulin as claimed in claim 14, which is in the form of an anti-sera composition or in the form of a monoclonal antibody.
16. The immunoglobulin of claim 14-15 which is capable of inhibiting the binding of Interleukin-12 to the Interleukin-12 receptor and is capable of neutralizing IL-12 bioactivity by binding to the IL-12 receptor.
- 15 17. The immunoglobulin of 14-15 which binds to the Interleukin-12 receptor but which is not capable of inhibiting the binding of Interleukin-12 to the Interleukin-12 receptor and is not capable of neutralizing Interleukin-12 bioactivity by binding to human IL-12 receptor.
- 20 18. A protein as claimed in any one of claims 8-13 for the binding or scavenge of Interleukin-12.
19. An immunoglobulin as claimed in any one of claims 14-17 for neutralising and/or inhibiting Interleukin-12 bioactivity.
- 25 20. A pharmaceutical composition comprising a low affinity Interleukin-12 receptor protein as defined in any one of claims 8-13 and a suitable diluent or carrier.
21. A pharmaceutical composition comprising an Interleukin-12 receptor protein as claimed in claim 20 containing one or more other cytokine receptor antagonists.
- 30 22. A pharmaceutical composition comprising an immunoglobulin as claimed in claim 14-17 and a suitable diluent or carrier.
23. A composition comprising human cells activated to express Interleukin-12 receptor bound with an immunoglobulin as claimed in claim 14-17, which immunoglobulin is labelled with a detectable label.
- 35 24. Use of an Interleukin- 12 receptor according to any one of claims 8-13 for the preparation of a pharmaceutical composition.
- 40 25. Use of an Interleukin-12 receptor according to claim 24 for the preparation of a pharmaceutical composition for treatment of diseases or conditions caused by an immune response to alloantigen and in the treatment of autoimmune dysfunction.
26. Use of an Interleukin-12 receptor protein as claimed in any one of claims 8-13 to bind or scavenge Interleukin-12.
- 45 27. Use of an immunoglobulin as claimed in claim 14-17 for the preparation of a pharmaceutical composition.
- 50 28. A process for the detection of an Interleukin-12 receptor which comprises isolating cells from a subject wich express the Interleukin-12 receptor, contatcing a sample of said cells with a detectable immunoglobulin capable of selectively binding to the Interleukin-12 receptor , incubating said cells under conditions which allow the detectable immunoglobulin to bind to the Interleukin-12 receptor, and detecting the binding of said cells to said immunoglobulin.
- 55 29. A process of claim 28 wherein the immunoglobulin is covalently bound to a solid resin and/or said immunoglobulin is labelled with a detectable label.

30. A process for the detection of soluble Interleukin-12 receptor which assay comprises capturing said receptor with an immunoglobulin capable of selectively binding to the IL-12 receptor and carrying out a binding assay with labelled Interleukin-12.

5 31. A method for determining an immune system abnormality in a subject which comprises determining the number of T cells, NK cells, or B cells in a sample from the subject, contacting the sample with an immunoglobulin capable of forming a complex with the human IL-12 receptor so as to form a cellular complex between the human IL-12 receptor and the immunoglobulin, determining the percentages of  
10 the T cells, NK cells, or B cells in the sample which have the human IL-12 receptor, comparing the percentages of said cells with the percentages of such cells from a normal subject who does not have the immune system abnormality, a difference in the percentages of the cells so determined being indicative of the immune system abnormality.

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Fig. 1

10	20	30	40	50	60	70
GGTGGCTGAA	CCTCGCAGGT	GGCAGAGAGG	CTCCCCCTGGG	GCTGTGGGGC	TCTACGTGGA	TCCGATGGAG
80	90	100	110	120	130	140
CCGCTGGTGA	CCTGGGTGGT	CCCCCTCCTC	TTCTCTTTCC	TGCTGTCCAG	GCAGGGCGCT	GCCTGCAGAA
150	160	170	180	190	200	210
CCAGTGAGTG	CTGTTTTCAG	GACCCGCCAT	ATCCGGATGC	AGACTCAGGC	TCGGCCTCGG	GGCCTAGGGA
220	230	240	250	260	270	280
CCTGAGATGC	TATCGGATAT	CCAGTGATCG	TTACGAGTGC	TCCTGGCAGT	ATGAGGGTCC	CACAGCTGGG
290	300	310	320	330	340	350
GTCAGCCACT	TCCTGCGGTG	TTGCCTTAGC	TCCGGGCGCT	GCTGCTACTT	CGCCGCCGGC	TCAGCCACCA
360	370	380	390	400	410	420
GGCTGCAGTT	CTCCGACCAG	GCTGGGGTGT	CTGTGCTGTA	CACTGTCACA	CTCTGGGTGG	AATCCTGGGC
430	440	450	460	470	480	490
CAGGAACCAG	ACAGAGAAGT	CTCCTGAGGT	GACCCTGCAG	CTCTACAACT	CAGTTAAATA	TGAGCCTCCT
500	510	520	530	540	550	560
CTGGGAGACA	TCAAGGTGTC	CAAGTTGGCC	GGGCAGCTGC	GTATGGAGTG	GGAGACCCCG	GATAACCAGG
570	580	590	600	610	620	630
TTGGTGCTGA	GGTGCAGTTC	CGGCACCGGA	CACCCAGCAG	CCCATGGAAG	TTGGGCGACT	GCGGACCTCA
640	650	660	670	680	690	700
GGATGATGAT	ACTGAGTCCT	GCCTCTGCCC	CCTGGAGATG	AATGTGGCCC	AGGAATTCCA	GCTCCGACGA
710	720	730	740	750	760	770
CGGCAGCTGG	GGAGCCAAGG	AAGTTCCTGG	AGCAAGTGGA	GCAGCCCCGT	GTGCGTTCCT	CCTGAAAACC
780	790	800	810	820	830	840
CCCCACAGCC	TCAGGTGAGA	TTCTCGGTGG	AGCAGCTGGG	CCAGGATGGG	AGGAGGCGGC	TGACCCTGAA
850	860	870	880	890	900	910
AGAGCAGCCA	ACCCAGCTGG	AGCTTCCAGA	AGGCTGTCAA	GGGCTGGCGC	CTGGCACGGA	GGTCACTTAC
920	930	940	950	960	970	980
CGACTACAGC	TCCACATGCT	GTCTTGCCCG	TGTAAGGCCA	AGGCCACCAG	GACCCCTGCAC	CTGGGGAAGA
990	1000	1010	1020	1030	1040	1050
TGCCCTATCT	CTCGGGTGCT	GCCTACAACG	TGGCTGTCTAT	CTCCTCGAAC	CAATTTGGTC	CTGGCCTGAA
1060	1070	1080	1090	1100	1110	1120
CCAGACGTGG	CACATTCTCTG	CCGACACCCA	CACAGAACCA	GTGGCTCTGA	ATATCAGCGT	CGGAACCAAC
1130	1140	1150	1160	1170	1180	1190
GGGACCACCA	TGTATTGGCC	AGCCCGGGCT	CAGAGCATGA	CGTATTGCAT	TGAATGGCAG	CCTGTGGGGC
1200	1210	1220	1230	1240	1250	1260
AGGACGGGGG	CCTTGCCACC	TGCAGCTGA	CTGCGCCGCA	AGACCCGGAT	CCGGCTGGAA	TGGCAACCTA
1270	1280	1290	1300	1310	1320	1330
CAGCTGGAGT	CGAGAGTCTG	GGGCAATGGG	GCAGGAAAAG	TGTTACTACA	TTACCATCTT	TGCCTCTGCG



Fig. 1 Cont.

1340	1350	1360	1370	1380	1390	1400
CACCCCGAGA	AGCTCACCTT	GTGGTCTACG	GTCCTGTCCA	CCTACCACTT	TGGGGGCAAT	GCCTCAGCAG
1410	1420	1430	1440	1450	1460	1470
CTGGGACACC	GCACCACGTC	TCGGTGAAGA	ATCATAGCTT	GGACTCTGTG	TCTGTGGACT	GGGCACCATC
1480	1490	1500	1510	1520	1530	1540
CCTGCTGAGC	ACCTGTCCCG	GCGTCCTAAA	GGAGTATGTT	GTCCGCTGCC	GAGATGAAGA	CAGCAAACAG
1550	1560	1570	1580	1590	1600	1610
GTGTCAGAGC	ATCCCGTGCA	GCCACAGAG	ACCCAAGTTA	CCCTCAGTGG	CCTGCGGGCT	GGTGTAGCCT
1620	1630	1640	1650	1660	1670	1680
ACACGGTGCA	GGTGCGAGCA	GACACAGCGT	GGCTGAGGGG	TGTCTGGAGC	CAGCCCCAGC	GCTTCAGCAT
1690	1700	1710	1720	1730	1740	1750
CGAAGTGCA	GTTTCTGATT	GGCTCATCTT	CTTCGCCTCC	CTGGGGAGCT	TCCTGAGCAT	CCTTCTCGTG
1760	1770	1780	1790	1800	1810	1820
GGCGTCCTTG	GCTACCTTGG	CCTGAACAGG	GCCGCACGGC	ACCTGTGCCC	GCCGCTGCCC	ACACCCTGTG
1830	1840	1850	1860	1870	1880	1890
CCAGCTCCGC	CATTGAGTTC	CCTGGAGGGA	AGGAGACTTG	GCAGTGGATC	AACCCAGTGG	ACTTCCAGGA
1900	1910	1920	1930	1940	1950	1960
AGAGGCATCC	CTGCAGGAGG	CCCTGGTGGT	AGAGATGTCC	TGGGACAAAG	GCGAGAGGAC	TGAGCCTCTC
1970	1980	1990	2000	2010	2020	2030
GAGAAGACAG	AGCTACCTGA	GGGTGCCCCCT	GAGCTGGCCC	TGGATACAGA	GTTGTCCTTG	GAGGATGGAG
2040	2050	2060	2070	2080	2090	2100
ACAGGTGCAA	GGCCAAGATG	<u>TGATCGTTGA</u>	GGCTCAGAGA	GGGTGAGTGA	CTCGCCCCGAG	GCTACGTAGC

Fig. 2

10	20	30	40	50	60	70
<u>MEPLVTWVVP LLEFLLSRQ GAACRTSECC FODPPYPDAD SGSASGPRDL RCYRISSDRY ECSWQYEGPT</u>						
80	90	100	110	120	130	140
AGVSHFLRCC LSSGRCCYFA AGSATRLQFS DQAGVSVLYT VTLWVESWAR NQTEKSPEVT LQLYNSVKYE						
150	160	170	180	190	200	210
PPLGDIKVS LAGQLRMEWE TPDNQVGAEV QFRHRTSPSS WKLGDCGPQD DDTESCLCPL EMNVAQEFQL						
220	230	240	250	260	270	280
RRRQLGSQGS SWSKWSSPVC VPPENPPQPQ VRFSVEQLGQ DGRRLTLKE QPTQLELPEG COGLAPGTEV						
290	300	310	320	330	340	350
TYRLQLHMLS CPCKAKATRT LHLGKMPYLS GAAYNVAVIS SNQFGPGLNQ TWHIPADTHT EPVALNISVG						
360	370	380	390	400	410	420
TNGTTMYWPA RAQSMTYCIE WQPVGQDGG L ATCSLTAPQD PDPAGMATYS WSRESGAMGQ EKCYYITIFA						
430	440	450	460	470	480	490
SAHPEKLT LW STVLSTYHFG GNASAAGTPH HVSVKNHSLD SVSVDWAPS LSTCPGVLR YVVRCRDEDS						
500	510	520	530	540	550	560
KQVSEHPVQP TETQVTL SGL RAGVAYTVQV RADTAWLRGV WSQPORFSIE VOVSDWLIFF ASLGSFLSIL						
-----						
570	580	590	600	610	620	630
<u>LVGVLYLGL NRAARHLCPP LPTPCASSAI EFPGGKETWQ WINPVDFQEE ASLQEALVVE MSWDKGERTE</u>						
-----						
640	650	660				
PLEKTELPEG APELALDTL SLEDGDRCKA KM						

Fig. 3

```

IL-12R GSASGI LR CY.RISSORY EGSWQYEGPT AGV.....
GP130 LPPEKPKNLS CYN.EGKXN RCEWGGGRET HLETNFTLKS .....EWA
G-CSF-R YPPAIPHNLS CLKNLTSSSL ICQWEPGPET HLPTSFTLKS FKSRGNCQTO
LIF-R YPPDTPQQLN CETH.DLKEI ICWNPGRVT ALVGP.RATS YTLVESFSCK
CONS .....P..L. C.....C.W.....T .....

SHFLRCC... LSSGRCCYFA AGSATRLQFS DQAGVSVLY. TVTLWVESWA
THKFADCKAK RDTPTSCTVD YSTVYFVNI. ....EVWVEAEN
GDSILDVCPK DGQSHCCIPR KHLLEYQNM. ....GIWVQAEH
YVRLK.....R AEAPTNESYQ LLFQMLPNQE IYNFTLNAHN
.h....C.....C.....Vve...

RNQTEKSPEV TLQLYNSVKY EPP.....L GDIKVSCLAG QLRMEWETPD
ALGKVTSDHI NFDVPYKVKP NPPHNLSVIN SEE....LSS ILKLTWTNPS
ALGTSMSPL CLDFMDVVKL EPPHLRTHDP SPEAAPPQAG CLQLCWEPWQ
PLGRSQSTIL VNITEKVYP HTPTSFKVKD INSTA....VKLSWHLPQ
.....S.....VK..pp.....l..l...W..p.

NOVGAEVQF..RHRT.PSSP WKLGDGCPQD .DDTESCLCP LEMNVAQEFO
IKSVIILKYN IQYRTKDAST W..SQIPPED TASTRSSFTV QOLKPFTEYV
PGLHINQKCE LRHKPQGEA SWALVGPLPL EALQYELCGL LPATAYTLQI
NFAKINFLCE IEIKSNSVQ EQRNVTIKGV ENSSYLVALD KLNP.YTLYT
.....rt...s. w.....p.d .....t.s.....e...

LR.RRQLGSQ GSSWSKNWSP VC.VPPENPP QPQVRFVSEQ LGQDGRRLT
FRIRCHKEDG KGYWSDWSEE ASGITYEDRP SKAPSFYKI DPSHTOGYRT
RCIRWPLPGH ...WSDWSPS LELRTTERAP TVRLDTWWRQ RQLOP...RT
FRIRSTETP W.KWSKWSNK KQHLTTEASP SKGPDW.RE WSSDG...KN
.r.R.....WS.WS...E..P .....f.....t

LKEOPTQLEL PEGCOGLAPG TEVYRLQLH MLSCPCAKA TRTLHLGKOP
VOLVWKT...L PPFANGKIL DYEVLTRWK SHLQNTV...NATKLTV
VOLFWKP..V PLEEDSGRIQ GYVVS...WRP SQQAGAILPL CNTTELSCTP
LIIYWKP..L PINEANGKIL SYNWSC...S SDEETQSLSE IPDPQHKAZI
.....l P.....SDEETQSLSE IPDPQHKAZI

YLSGAAYNVA VISSNQPCPG LNQTWHIPAD T...HTEPVA LNISVGTNGT
NLTDNDYLAT LTVRNLVGKS DAAVLTIPAC DFOATHPVMD LKAPFKDMKL
HLPSEAQEVA LVAYNSAGTS RPTPVVFSZ R..GPALTRL HAMARDPHSL
RLDKNDYIIS VVAJNSVGSS PPSKIASMEI P..NDDLKIE QVVGMG.KGI
.L....y....N..G...ips.....l.....n..

THYW.PARAQ SHTYCIWOP VQDGGGLATC SLTAPQDPDP AGMATYSWSR
WVEWTTPRES VKYIILENCV LSDK...APC .ITDWOQEDG TVHRTYLR...
WVGWEPNPM POGYVIEGL GPPSASNSK TWRMDONGRA TG...FLIKE
LLTWHYDPNM TCDYVIRWCN SSRS...EPC LMDWRKVPSN STETVI...
...W...r....Y..ew...a.c ..t..q..d...ty...

ESGANGQEK C YITIFASAH PEKLTWSTV LSTYHFGGNA SAAGTPHVS
..GNLAESKC YLITV.TPVY ADGPG.SPES IKAYL..KQA PPSKGT.VR
NIRPPQ...L YEIIIVPLYQ DTM...GPS QHVVAYSQEM APSHAP.ELH
ESDEPRGIR YNFFLYGCRN QGYQLRSMI ..GY..IEEL APIVAP.NFT
..g.....kc Y.it.....Y.....a .....P..v.

VONHSLDSVS VOWAPSLST CPGVLKEYV. ....VRCRDEDS
TKRVGNZAV LENDQLPVDV QNGFIRNYTI FYRTIIGNET AVNV.....
LKHIGKTAQ LENVPEPPEL GKSPLTHYTI FWTNAQNSP SAIL.....
VEDTSADIL VKWEDIPVEE LRGLRGLYF YFGKGERDTS KQVLESGRS
.k.....W.....g....Y.. .....

KQVSEHPVQ TETQVTLSSL RAGVAYTVQV RADTAWLRGV WSQPO...RFS
.....DS SHTFYTLSSL TSDTYLNVRM AAYTDEGGK GPEFTTTPK
.....NA SSRGFLHGL EPASLYHML MAASQAGATN STVLTMLTL
DIKVNITDI SOKTLRIADL QGKTSYHLVL RAYTGGGVGP EKSXYVTKZ
.....t..tis.L .....Y.v...A.t.....

IEVQVSDWLI FFASLGSFLS ILLVGVLYL GLNR..AARH LCPPLPTPCA
FAQGEIEAV VPVCLAFLLT TLLGVLF..C FNRDLIKH IWPVNDPSK
PEGSZLHIL GLFGLLLLT CLCGTAWLCC SPNR...KNP LWPSVDPAP
NSVGLIIAIL IPVAVAVIG VVTS...ILC YRKREWIKET FYDIPNPN
.....l...l..ll.....R.....h ..P..P..P..

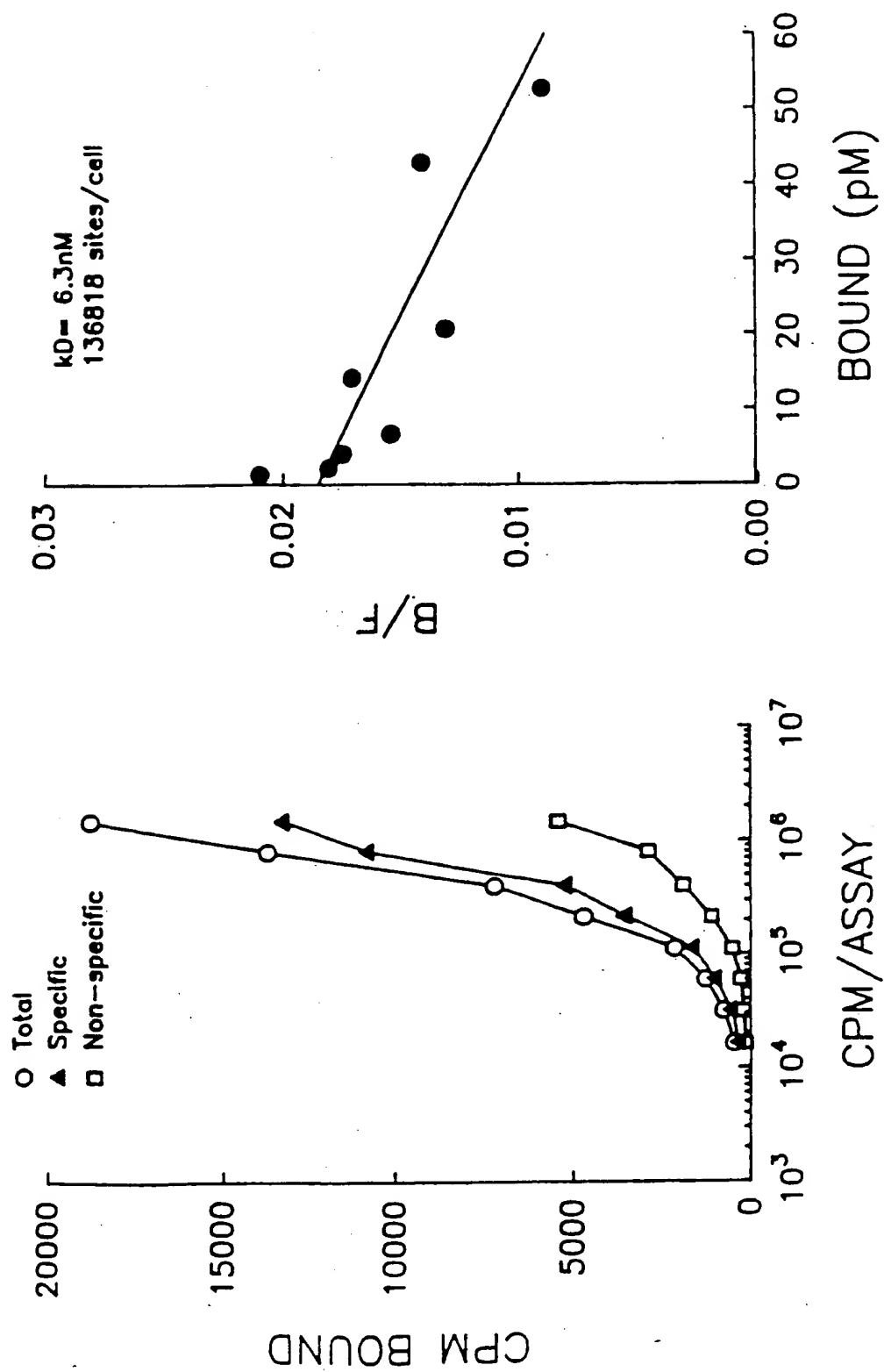
SSAIEPPGGK ETWQWNPVD .FQEEASLQZ ALVVEN.SWD KGERTEPLEK
SHIAQWSPHT PPRHNFNSKD QMYSDCNFTD VSVVEIAND KKPFPDLKS
SSLGSWVPTI MZEDAPQLPG L..GTFPIK LTVLEDEKK PVPW.ESHNS
CKALQPKSV CEGSS.ALKT LEMNPCTPNW VEVLETRAS PK.I.EDTEI
s.....n..d .....VVE....d k....E..l..

TELEGAPEL ALDELSLED GDRCKAOM..
LDLFX.KEXI NTEGHSSCIG GSSCHSSSRP S
SETCGLPTLV QTYVLOGDPR AVSTQPSQS
ISPVAERPED RSDAEPENW VVSYC
..l.....s....g..c.....

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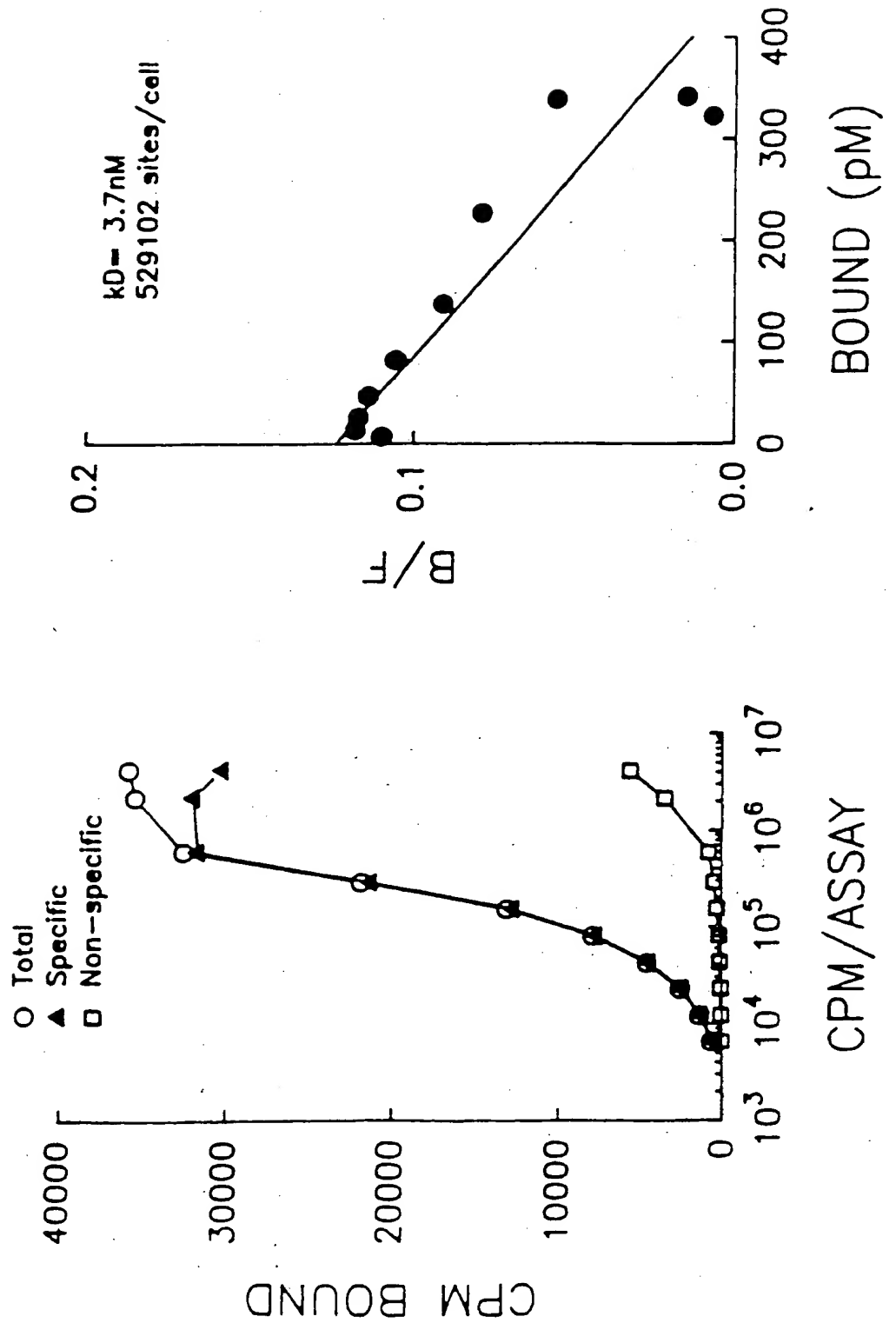
## COS(H12R) CELLS: [125]-I-HU-IL-12 BINDING

Fig. 4 a



# COS(H12R) CELLS: [125]-I-24E6 BINDING

Fig. 4 b



**Fig. 5**

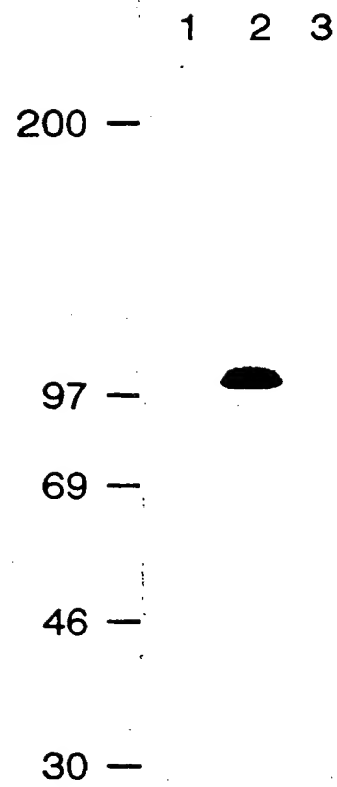


Fig. 6

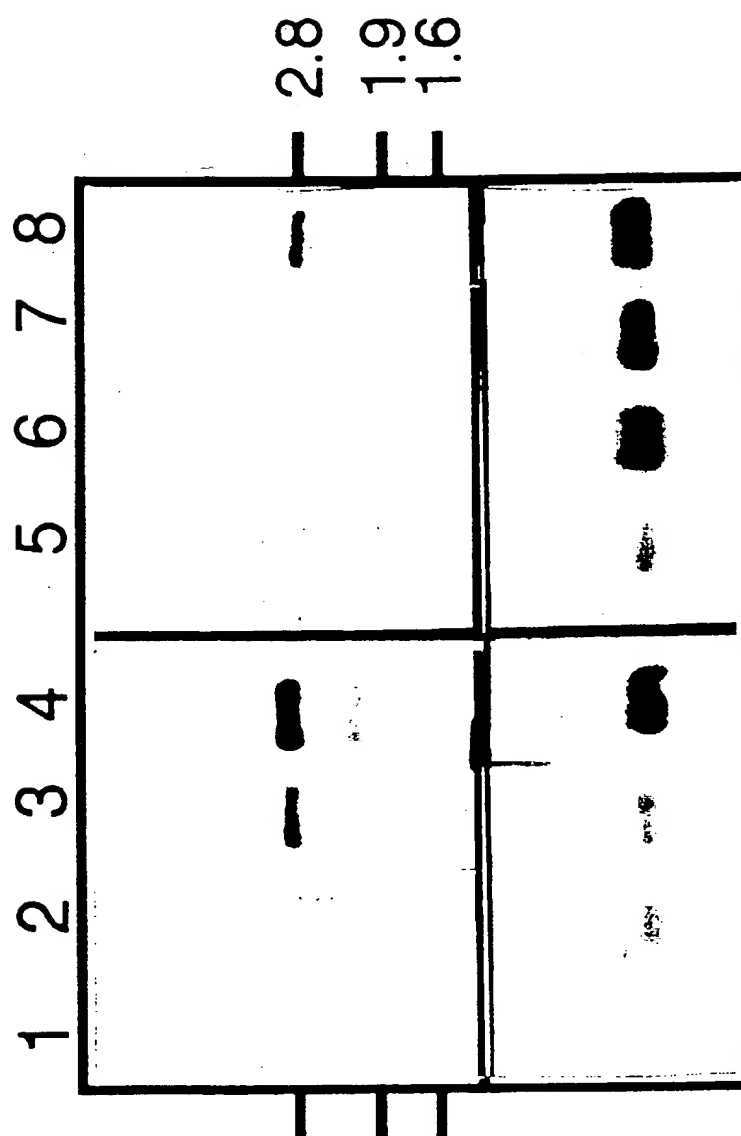


Fig. 7

INHIBITION OF  $[^{125}I]$ -IL-12 RECEPTOR BINDING BY  
MOUSE ANTI IL-12 RECEPTOR ANTISERUM

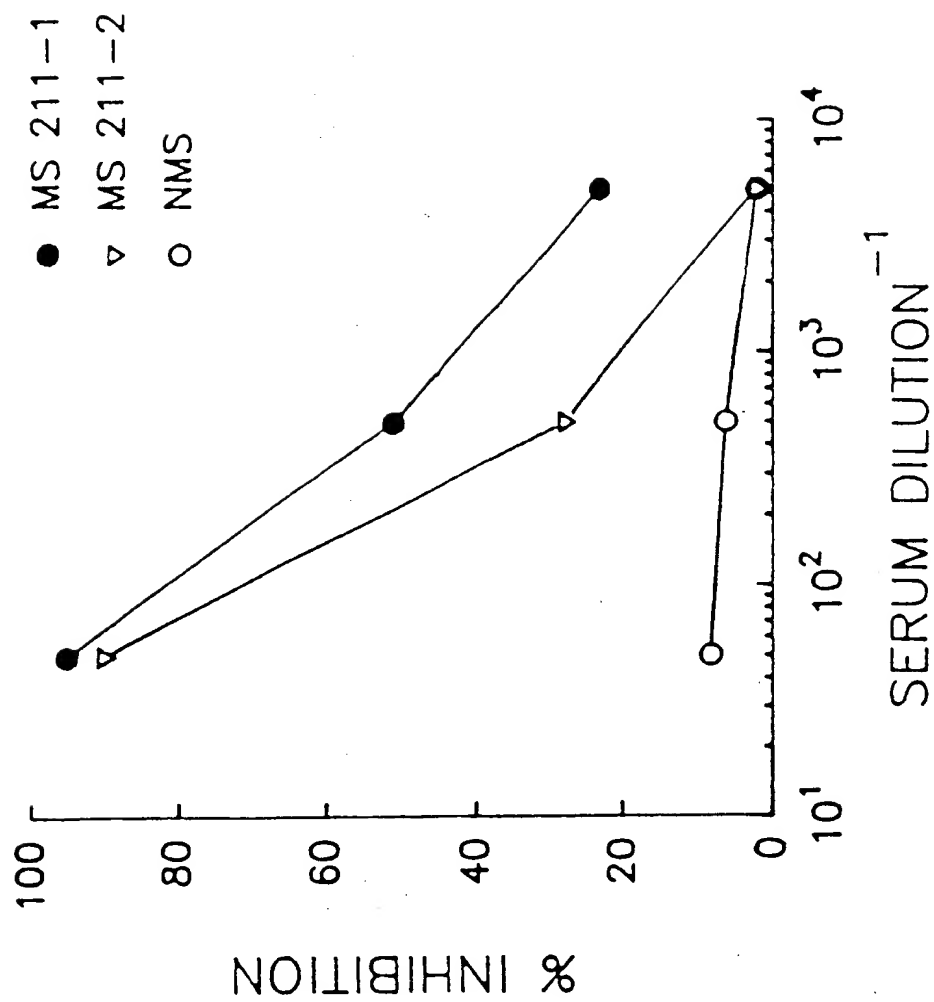




Fig. 8

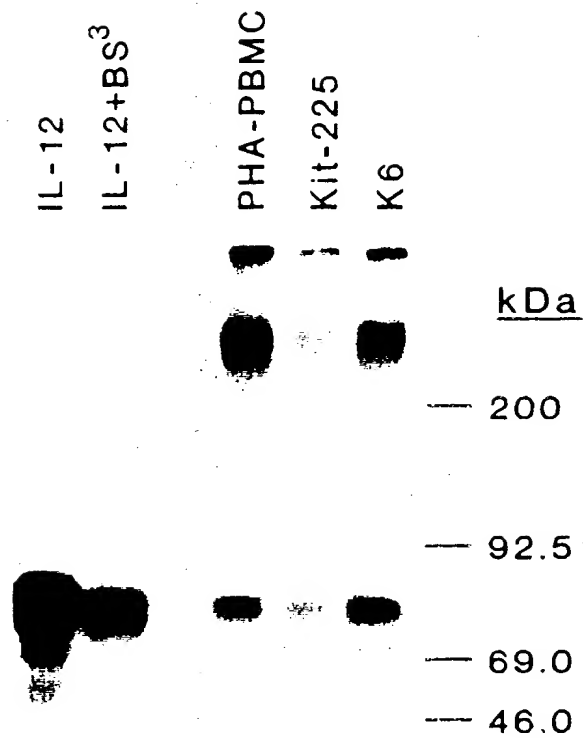


Fig. 9

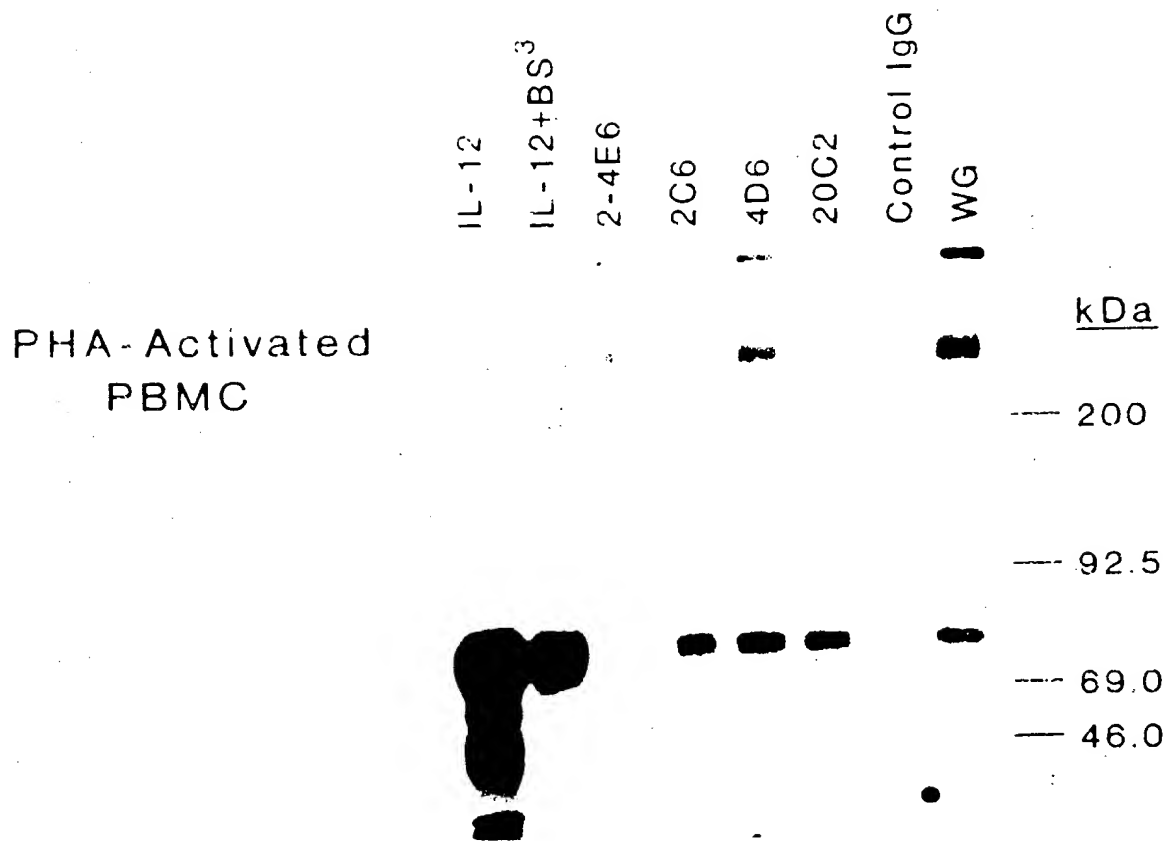


Fig. 10

PHA-PBLS: [125]-I-24E6 IgG BINDING

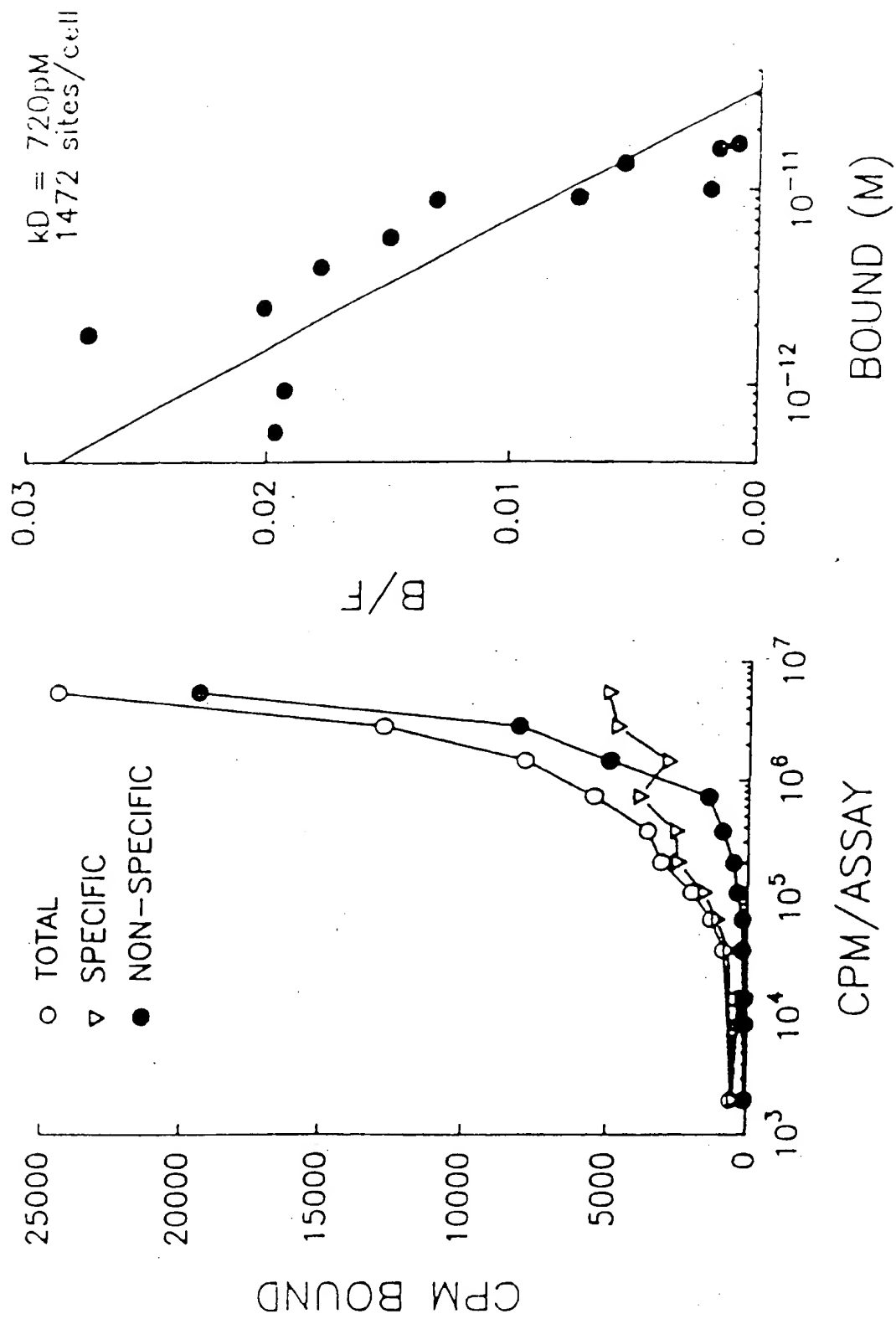


Fig. 11

## K6 CELLS: [125]-I-24E6 IgG BINDING

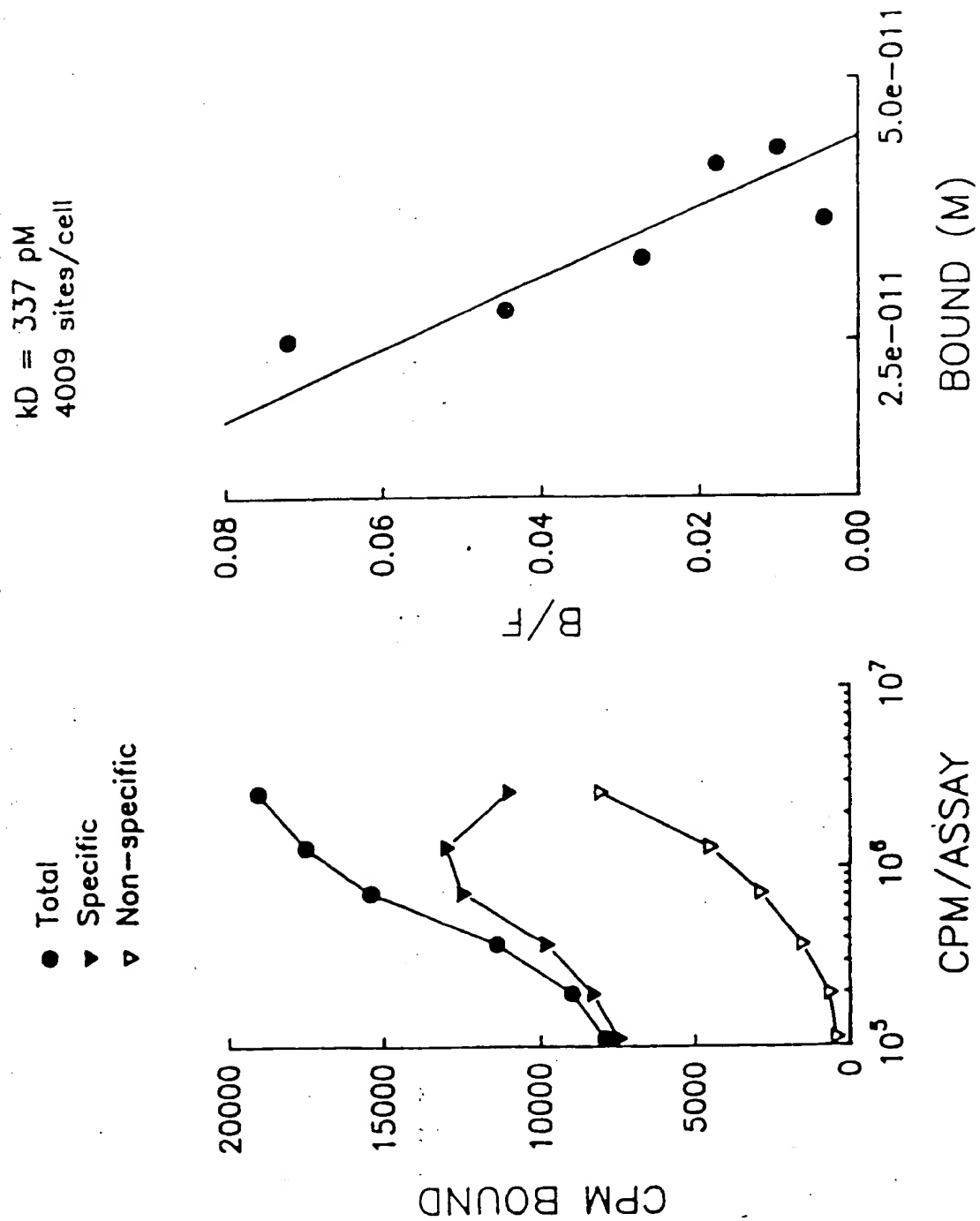


Fig. 10

## PHA-PBLS: [125]-I-24E6 IgG BINDING

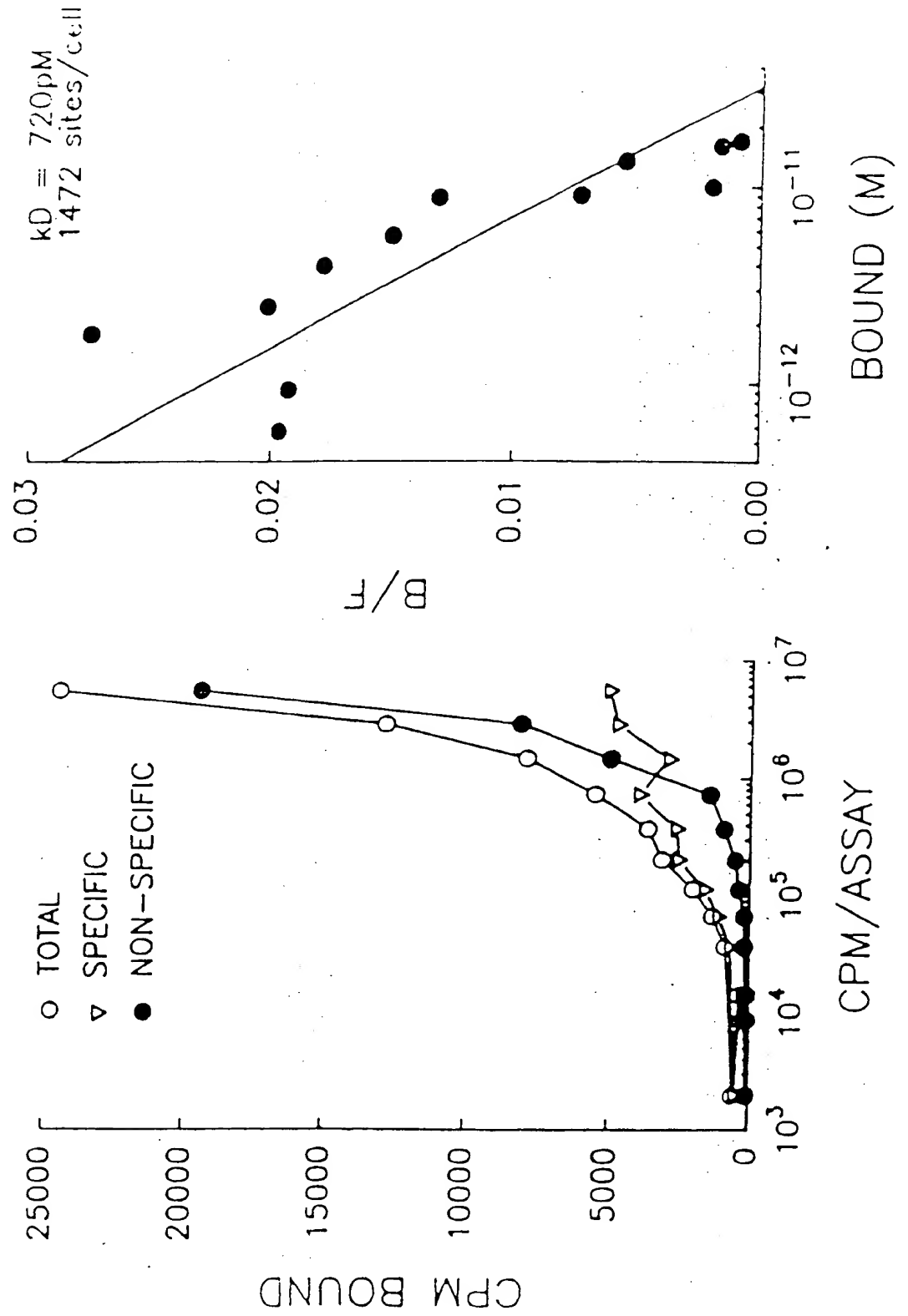


Fig. 11

## K6 CELLS: [125]-I-24E6 IgG BINDING

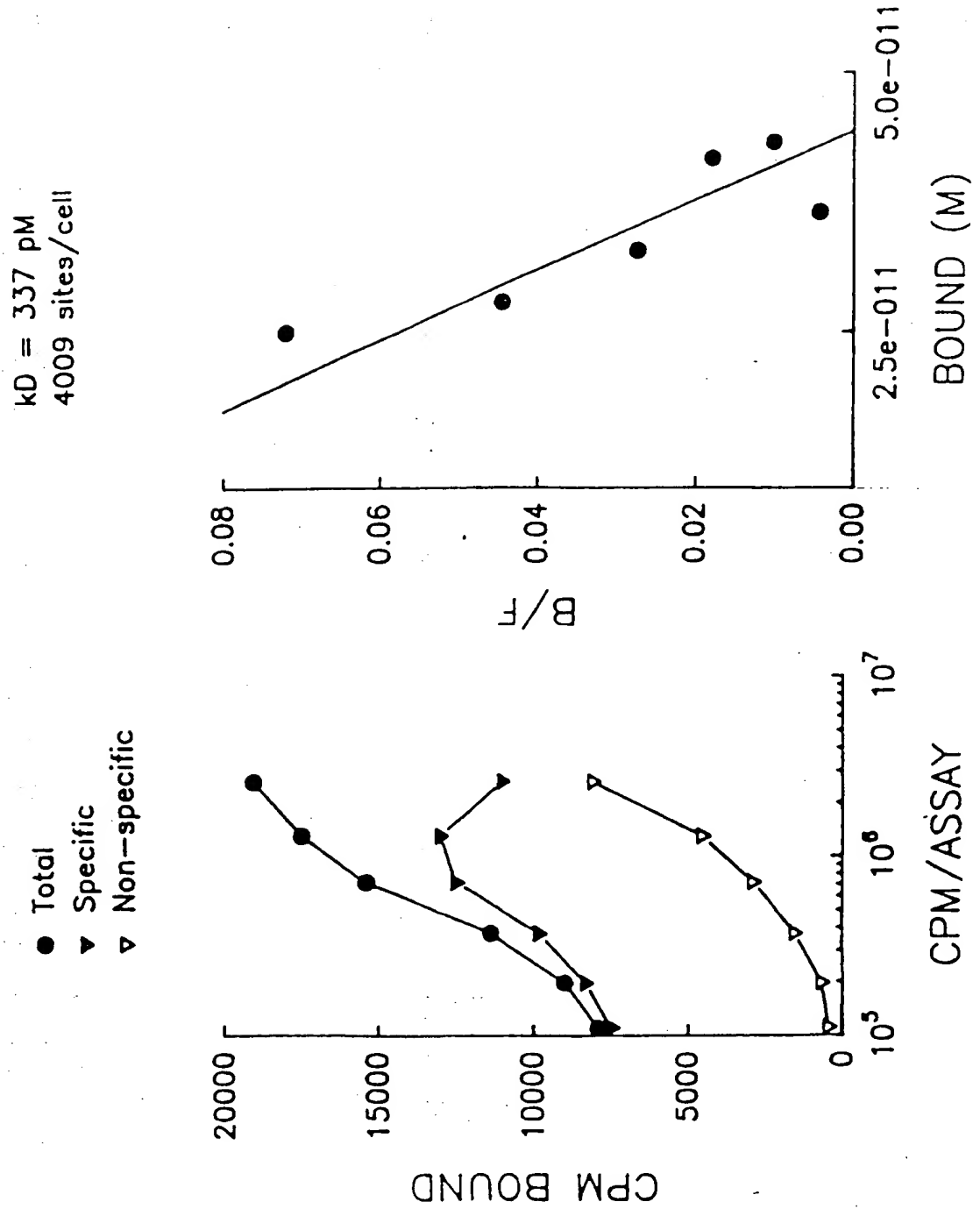


Fig. 12

## 125-I-24E6 COMPETITIVE BINDING TO K6 CELLS

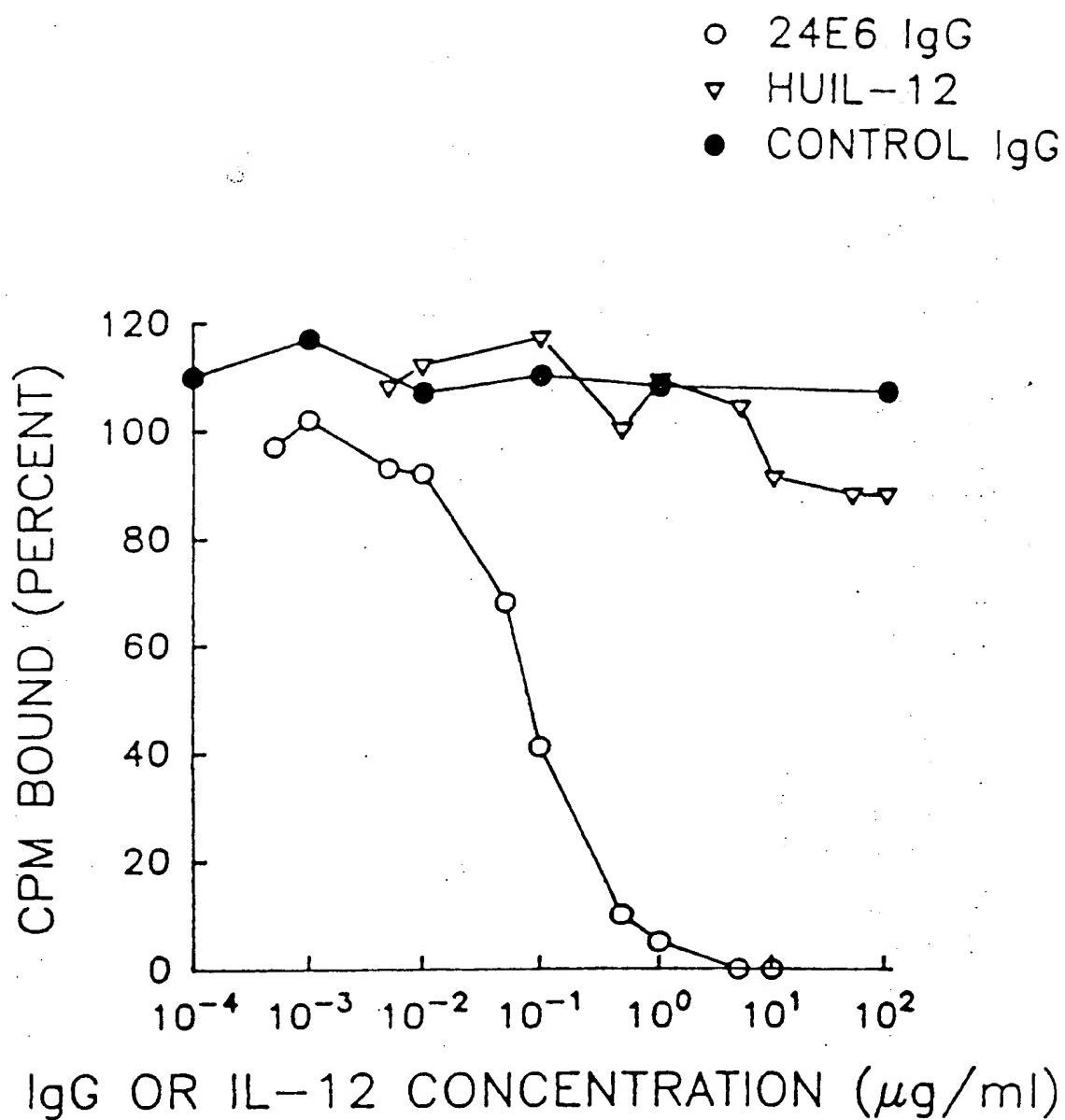


Fig. 13

## K6 CELLS: [125]-I-HUIL-12 (LSA) BINDING

kD = 16pM

707 sites/cell

kD = 57 pM

939 sites/cell

kD = 2.4nM

4036 sites/cell

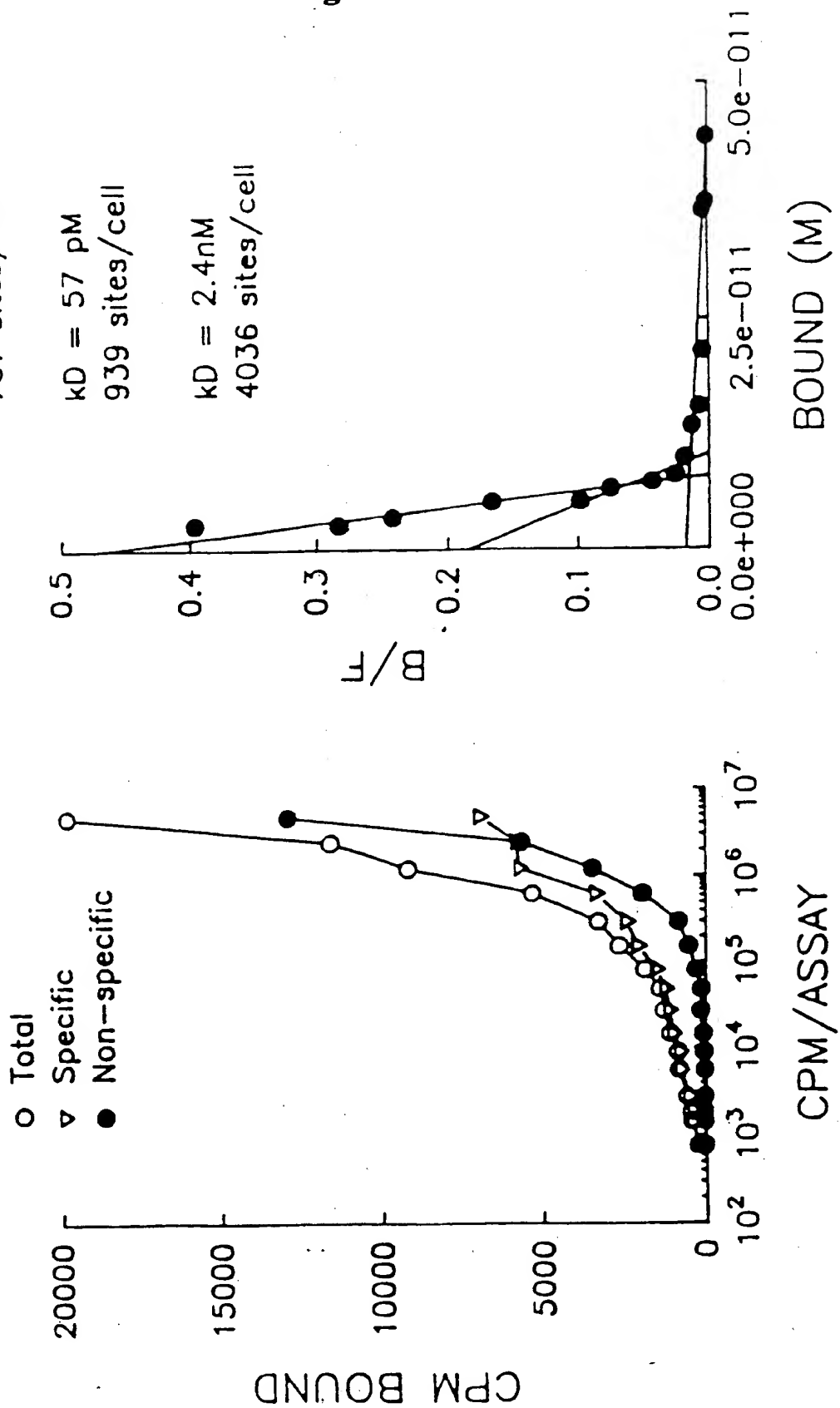
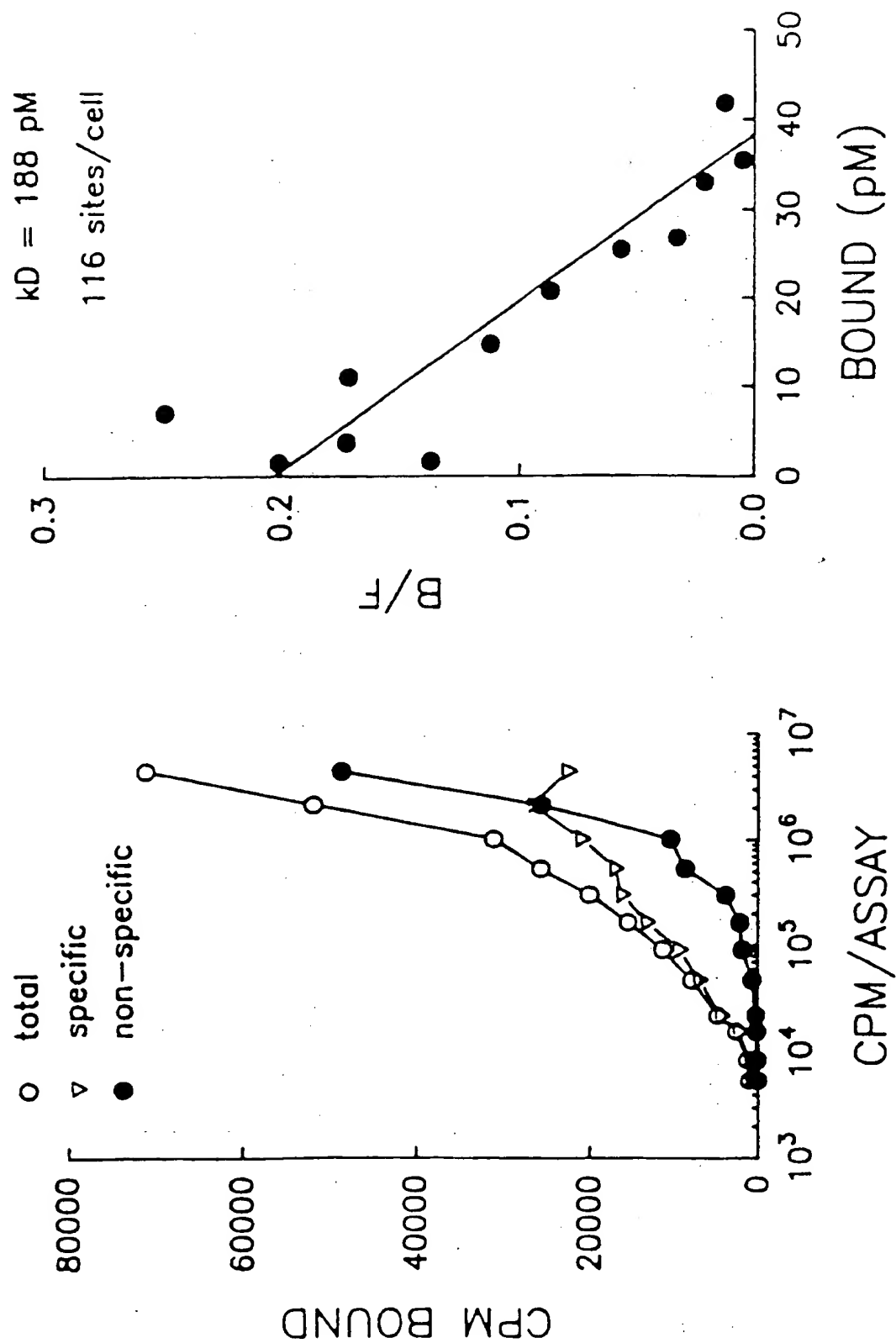




Fig. 14

## [125]-I-HUIL-12 BINDING TO K6 SOLUBLE RECEPTOR



**Fig. 15**

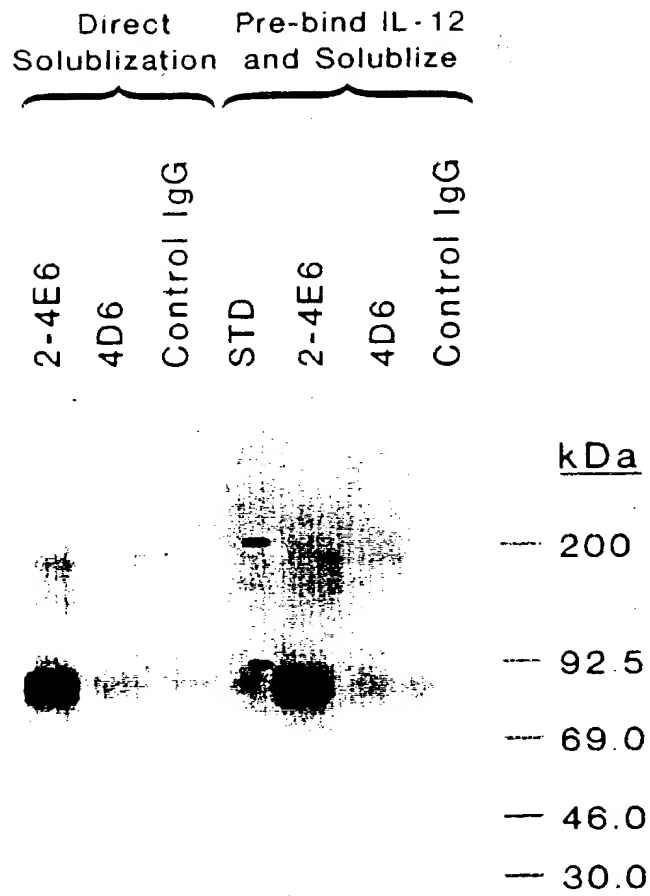


Fig. 16

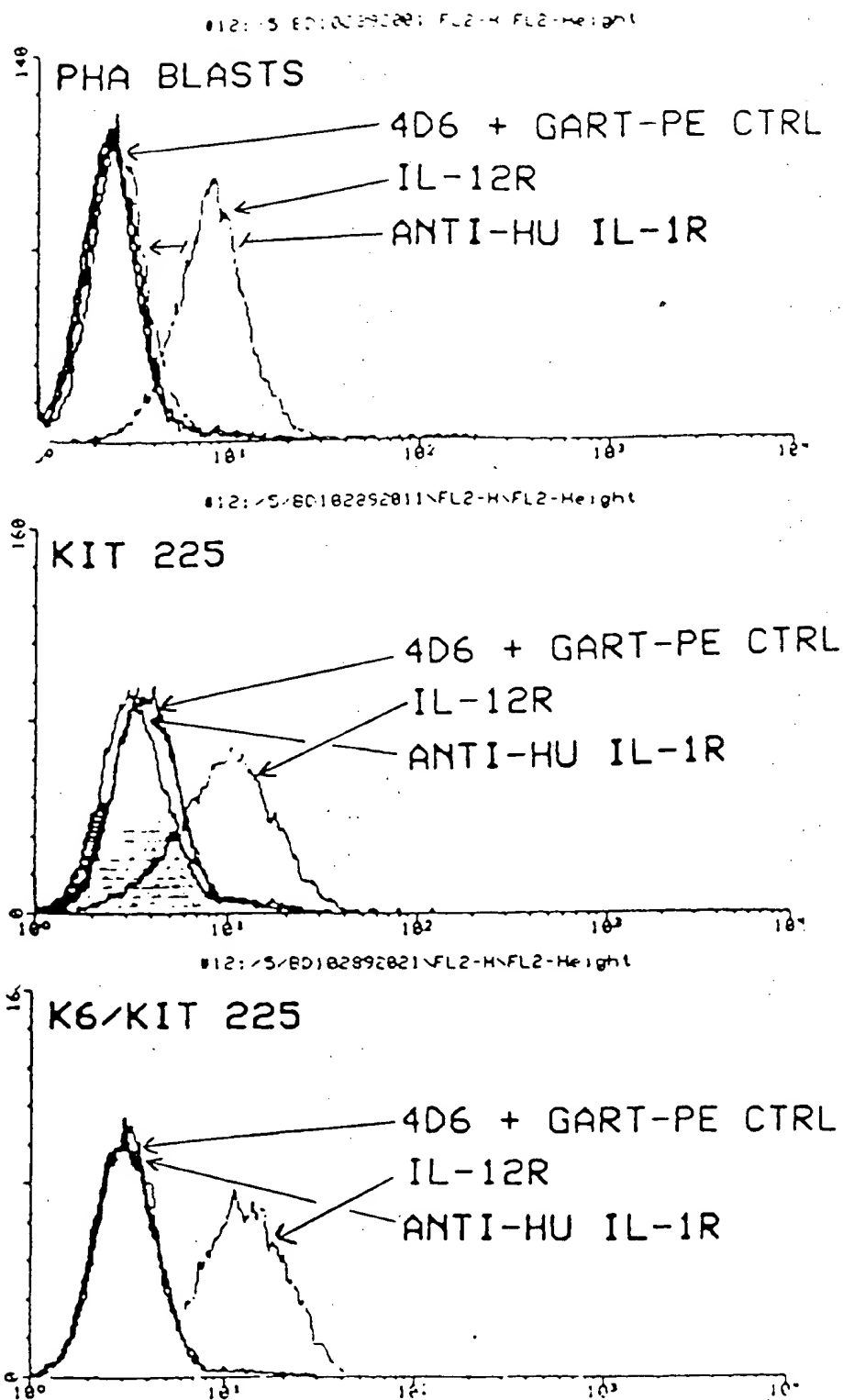


Fig. 17

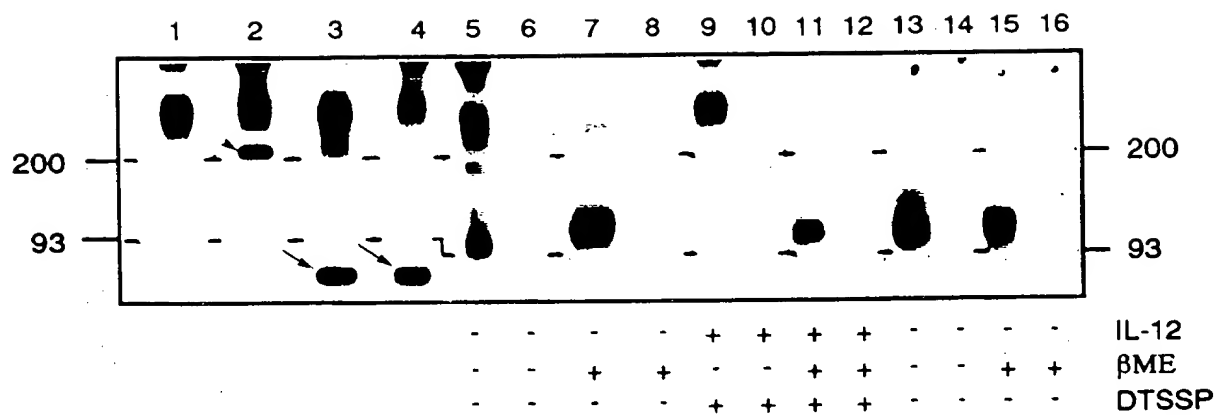


Fig. 18 a

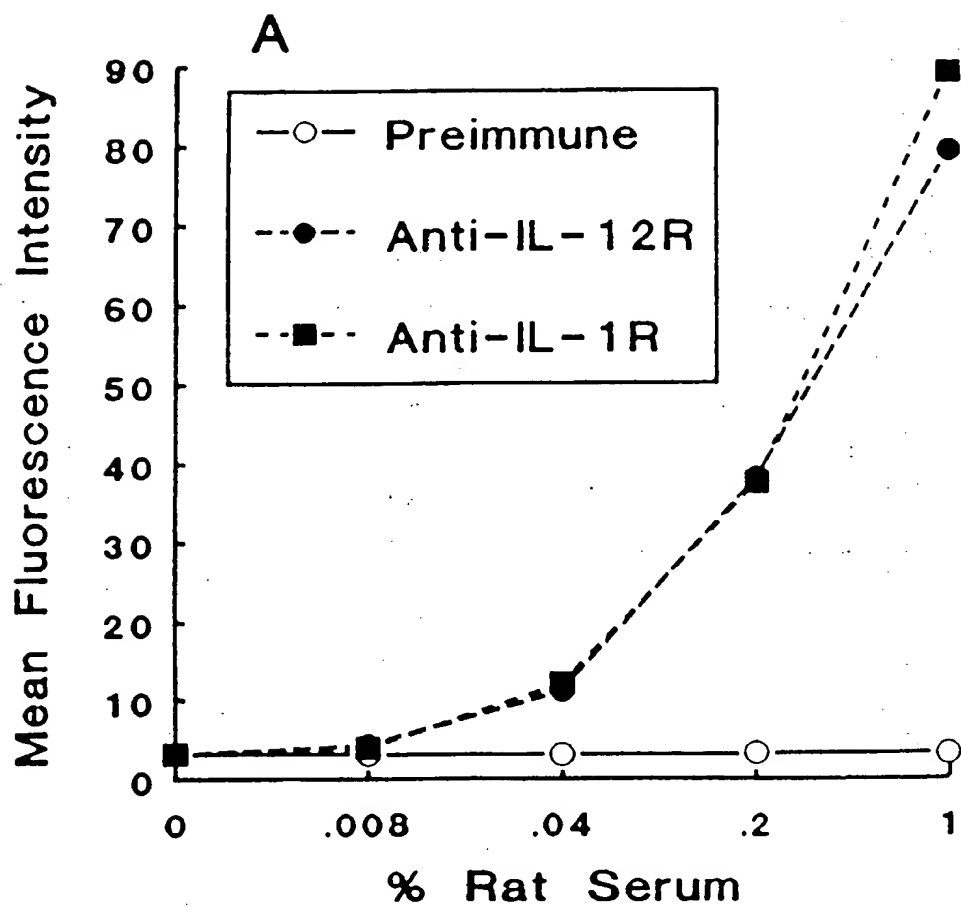


Fig. 18 b

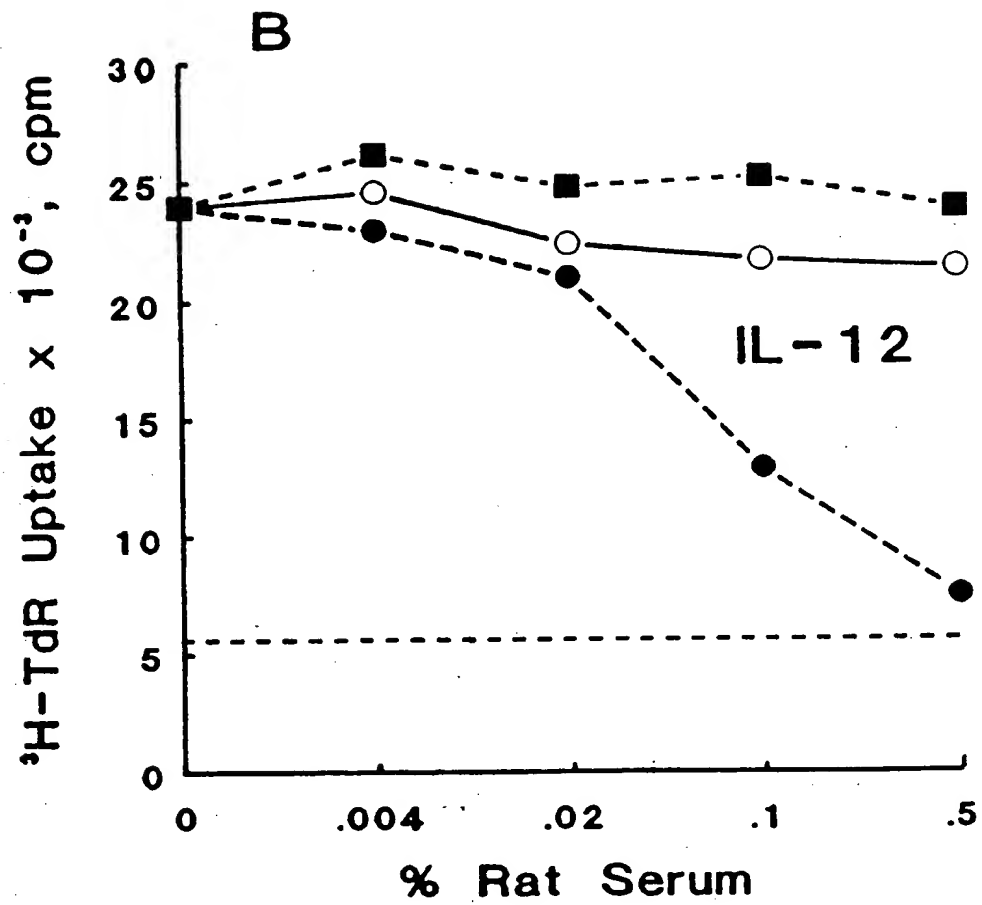


Fig. 18 c

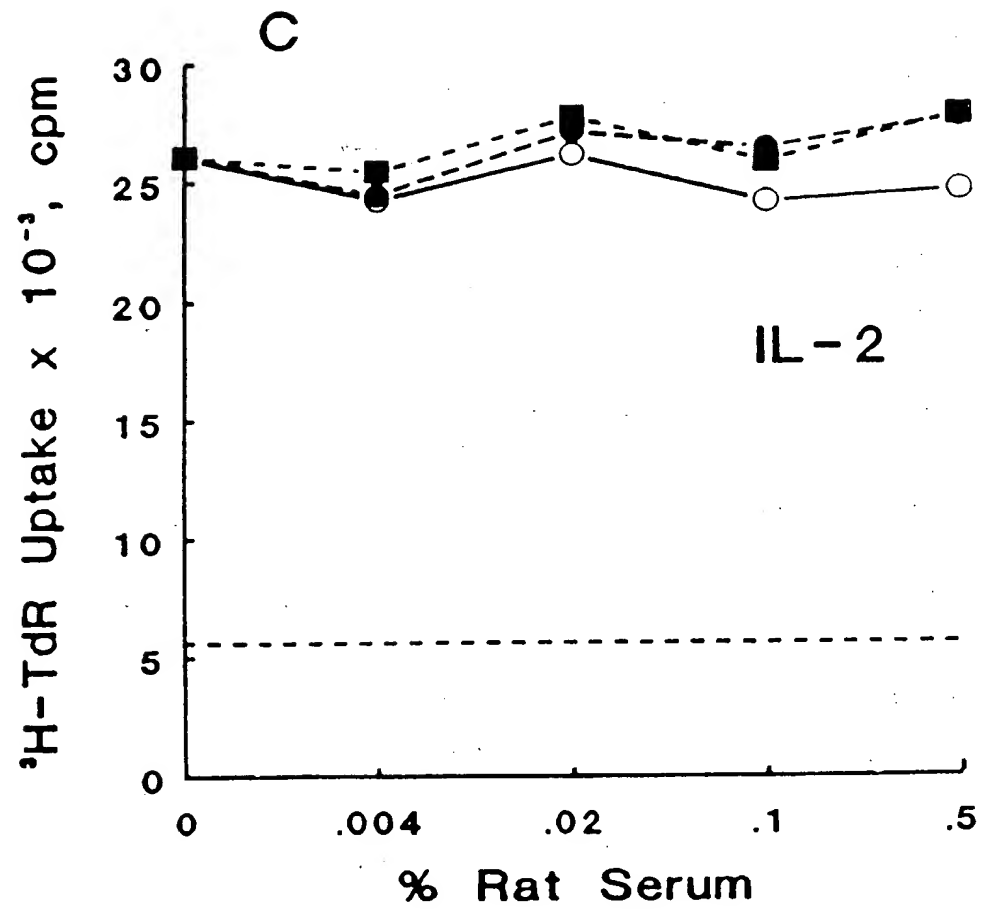


Fig. 18 d

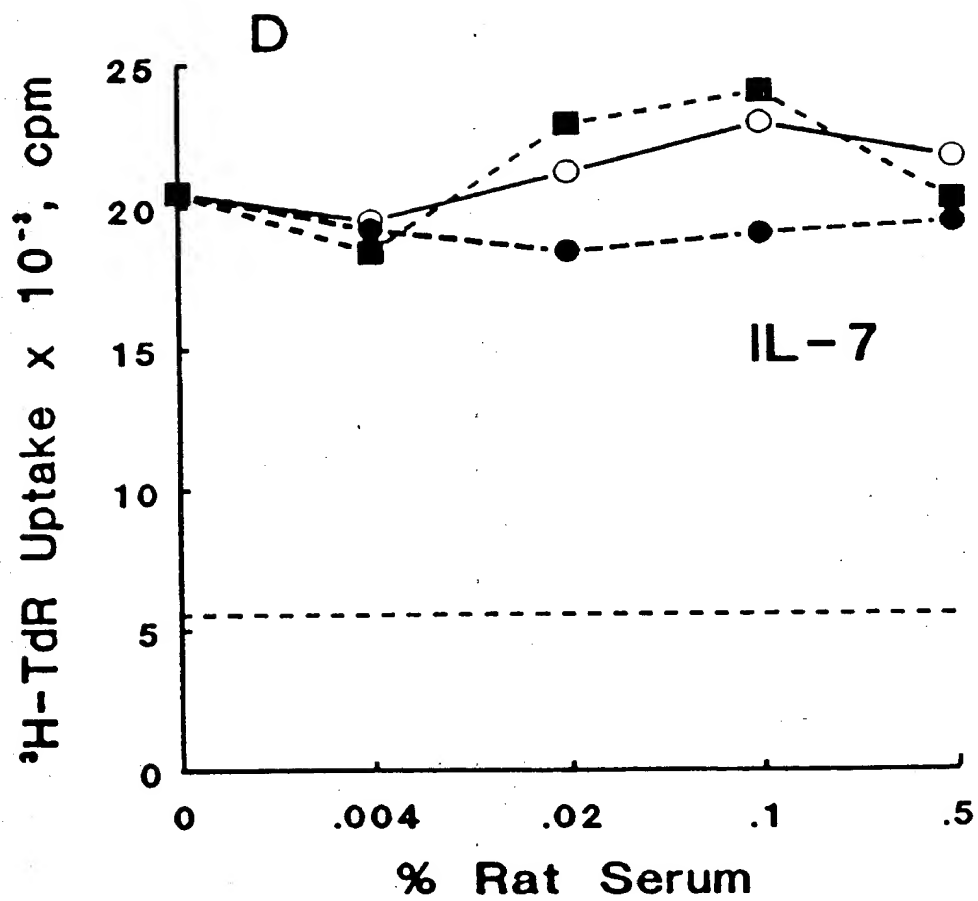
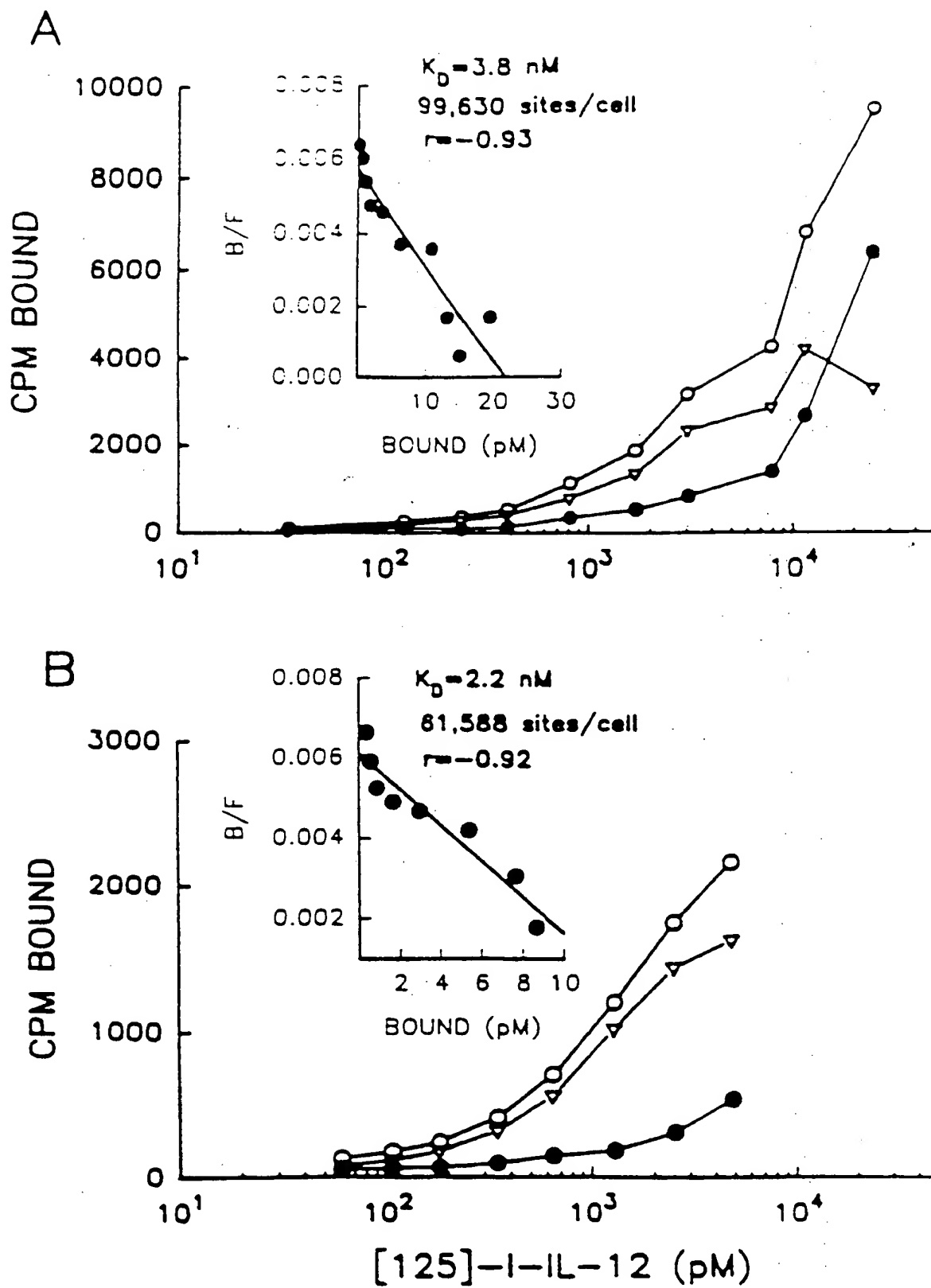




Fig. 19





European Patent  
Office

## EUROPEAN SEARCH REPORT

Application Number  
EP 94 11 0657

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
P, X	JOURNAL OF IMMUNOLOGY., vol.153, no.1, 1 July 1994, BALTIMORE US pages 128 - 136 CHUA AO;CHIZZONITE R;DESAI BB;TRUITT TP;NUNES P;MINETTI LJ;WARRIER RR;PRESKY DH;LEVINE JF;GATELY MK;ET AL ; 'Expression cloning of a human IL-12 receptor component. A new member of the cytokine receptor superfamily with strong homology to gp130.' * the whole document *	1-31	C12N15/12 C07K14/715 C07K16/28 A61K38/17 A61K39/395 G01N33/68
X	JOURNAL OF IMMUNOLOGY., vol.150, no.8, May 1993, BALTIMORE US page 270A DESAI B;TRUITT T;HONASOGE S;WARRIER R;CHIZZONITE R;GATELY M; 'EXPRESSION OF FUNCTIONAL IL-12R ON A HUMAN IL-2-DEPENDENT T CELL LINE' & JOINT MEETING OF THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS AND THE CLINICAL IMMUNOLOGY SOCIETY, DENVER, COLORADO, USA, MAY 21-25, 1993.	1-12, 20, 21, 24-26, 28-31	TECHNICAL FIELDS SEARCHED (Int.Cl.6)  C07K C12N A61K
D, A	JOURNAL OF IMMUNOLOGY., vol.148, no.10, 15 May 1992, BALTIMORE US pages 3117 - 3124 CHIZZONITE R;TRUITT T;DESAI BB;NUNES P;PODLASKI FJ;STERN AS;GATELY MK; 'IL-12 receptor. I. Characterization of the receptor on phytohemagglutinin-activated human lymphoblasts.' --- -/--	1-31	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 5 December 1994	Examiner NAUCHE, S
<b>CATEGORY OF CITED DOCUMENTS</b> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document  T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document			



European Patent  
Office

## EUROPEAN SEARCH REPORT

Application Number  
EP 94 11 0657

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,A	JOURNAL OF IMMUNOLOGY., vol.148, no.10, 15 May 1992, BALTIMORE US pages 3125 - 3132 DESAI, B.B. ET AL.; 'IL-12 receptor. II. Distribution and regulation of receptor expression' * the whole document *	1-31	
A	JOURNAL OF CELL BIOLOGY, vol.0, no.17, February 1993 page 73 CHIZZONITE R;TRUITT R;GRIFFIN M;NICKBARG E;HUBBARD B;DESAI B;STERN A;GUBLER U;GATELY M ; 'INITIAL CHARACTERIZATION OF THE IL-12 RECEPTOR IL-12R ON CONCAVALIN-A ACTIVATED MOUSE SPLENOCYTES' & KEYSTONE SYMPOSIUM ON CYTOKINES AND CYTOKINE RECEPTORS: FROM CLONING TO THE CLINIC, KEYSTONE, COLORADO, USA, JANUARY 31-FEBRUARY 7, 1993.	1-31	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 5 December 1994	Examiner NAUCHE, S
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X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document	

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